CANOLA PHENOLICS:

COLOR AND STRUCTURAL CHANGES OF

SINAPINE AND SINAPIC ACID

By

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A Thesis

Submitted to the Faculty of Graduate Studies in Partial Fulfillment

of the Requirements for the Degree of

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PREFACE

This thesis has been written in paper format. It consists of seven chapters. Chapter I is a literature review, covering canola composition, processing, chemistry, and scientific methods related to the research in this thesis. Chapter II to VI contain five research sections, covering structural changes and coloration of sinapic acid and sinapine during autoclaving, structural changes and coloration of sinapic acid during alkaline air oxidation, structural changes and coloration of sinapine during alkaline hydrolysis and air oxidation, HPLC determination of sinapine and sinapic acid, and the effect of processing conditions and the presence of sinapine and sinapic acid on the color of canola protein isolate. These chapters could be viewed as individual papers but as a whole they constitute the integral objectives for the thesis. An attempt was made to avoid duplication but some repetition was unavoidable in the introduction of each chapter to maintain the fluency of the papers. Chapter II and III have been published by Journal of the American Oil Chemists' Society, 76:433-441 and 76 :757-764, 1999. Chapter VII contains the appendixes, which offer supporting data for the individual papers.

ABSTRACT

Dark color is one of the major problems that hinders canola protein utilization. In order to assess the role of canola phenolics in determining the visual attributes of canola protein as affected by the processing conditions, several studies investigating the effect of processing conditions on the color and structural changes of canola phenolics have been undertaken. Investigations were made using pure phenolic systems and phenolic-protein systems of canola seed and meal. Two typical processing conditions, autoclaving and pH adjustment, were used as treatments in the current research. Structural changes of the phenolics were followed by techniques including high performance liquid chromatography (HPLC), spectral analysis, thin layer chromatography (TLC), nuclear magnetic resonance (NMR) and mass spectroscopy (MS). Color properties were determined by spectral analysis for liquids and by a HunterLab Color/Difference Meter for solid materials. Phenolic content was determined by both a newly developed HPLC method and a conventional spectral colorimetric method (Folin-Ciocalteu's reagent method).

Autoclaving was found to affect the visual properties of sinapic acid but not those of sinapine. The colorless sinapic acid solution turned yellow after a 15-minute autoclaving at 121 °C and 0.1 MPa. Filtering the solution through a 0.45-μm filter resulted in a brown solid consisting of at least three undetermined colored substances (yellow, orange and purple) and a yellow liquid. A yellow substance, syringaldehyde, has been identified in the yellow liquid by NMR, mass spectroscopy and HPLC.

The alkali induced air oxidation of sinapic acid at elevated pH values converted sinapic

acid to thomasidioic acid by a first order process. The resulting thomasidioic acid further oxidized to form 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid. Structural changes to sinapic acid during alkali induced air oxidation caused a darkening of the color for the system with the 2,6-dimethoxy-p-benzoquinone being one major color contributor. A similar study in the sinapine solution showed a more remarkable color darkening for the system due to the structural changes induced by air oxidation under alkaline conditions.

The movement of the research from pure phenolic systems to phenolic-protein systems required a rapid and reliable method for phenolic determination. A rapid HPLC method for sinapine and sinapic acid determination was developed, where sinapine and sinapic acid were separated using a gradient elution.

Processing conditions have been shown to affect the color of canola flour and protein isolate. Alkaline extraction produced a darker protein isolate than did aqueous NaCl extraction. Autoclaving darkened the canola flours. While the total soluble phenolic content decreased, the total insoluble-bound phenolic content increased after autoclaving. Sinapic acid increased the yellow intensity of canola protein during autoclaving while sinapine slightly decreased the lightness of canola protein. Autoclaving also decreased soluble phenolic content while increasing insoluble-bound phenolic content. Neither sinapic acid nor sinapine had any negative effect on the color of the canola protein during isolation using basic extraction with acidic precipitation.

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LIST OF ABBREVIATIONS

AAP = Aqueous Extraction with Acidic Precipitation

ANOVA = Analysis of Variance

BAP = Basic Extraction with Acidic Precipitation

CIE = Commission Internationale de L'Eclairage

HPLC = High Performance Liquid Chromatography

MS = Mass Spectroscopy

NMR = Nuclear Magnetic Resonance

PMM = Protein Micellar Mass

SA = Sinapic Acid

SPN = Sinapine

SPNB = Sinapine Bisulfate

TH = Total Phenolic Determined by HPLC

TF = Total Phenolic Determined by Folin-Ciocalteu reagent

TLC = Thin Layer Chromatography

UIS = Visual

UV = Ultraviolet

INTRODUCTION

Rapeseed/canola is one of the world's most important oilseed crops and in Canada it is second only to wheat in value and area planted (Shahidi and Naczk, 1992). Rapeseed/canola is used for the production of a high quality edible oil and a feed grade meal not normally used for food (Shahidi, 1990), despite the meal having a well balanced amino acid content and a favorable protein efficient ratio. Unsuitability for food is due to the presence of some undesired minor compounds, such as glucosinolates, phytates and phenolic compounds. The development of "canola" varieties by Canadian breeders has greatly reduced erucic acid content (to less than 1%) and glucosinolate content (to less than 15 μmol/g). However, the utilization of canola meal is still thwarted by the presence of phenolic compounds (Shahidi and Naczk, 1992). Phenolics have significant effects on the visual attributes, flavor characteristics, nutritional and even functional properties of oilseed products (Shahidi and Naczk, 1995). In addition, oilseed phenolics may have antioxidative properties (Swern, 1979; Shahidi et al., 1995; Shahidi and Warnasundara, 1992), and some phenolics may also have antimicrobial activity (Raccach, 1984). Sinapine in canola meal, for example, can cause bitter tasting, unpleasant flavor and dark color for the meal (Shahidi and Naczk, 1992). Sinapine was also the precursor of trimethylamine (TMA), which can cause the tainting of eggs when meal was used as poultry feed (Shahidi and Naczk, 1992). This lowers the nutritional value of the egg product. Chlorogenic acid, a phenolic in sunflower meal, can cause the discoloration of the meal and can also cause defects in the protein bio-availability

through binding with protein (Shahidi and Naczk, 1995). Gossypol in cottonseed, on the other hand, has been reported to have antioxidative properties for the seed products, especially for oil products (Swern, 1979). However, gossypol is toxic to monogastric animals and dark in color (Shahidi and Naczk, 1995). The *p*-coumaric acid in peanut also causes flavor and color defects for peanut protein, even when it is present at concentrations of 0.02-0.2% (Blair and Reichert, 1984), much lower than sinapine and chlorogenic acid, which can represent up to 2.5% and 2.7% of the meal (Shahidi and Naczk, 1995; Dorrell, 1976). Therefore, although the beneficial aspects, such as antioxidative properties, are recognized, oilseed phenolics have mainly detrimental effects on oilseed products.

In canola/rapeseed, sinapic acid and sinapine are the predominant phenolic compounds. The chemical and biochemical activities of these substances have received much attention. Studies on the metabolism of sinapic acid derivatives during seedling development of rape showed that the metabolic pathway of sinapine involved the synthesis of sinapoylmalate through sinapoylglucose (Bouchereau et al., 1992). In addition, the concentration of sinapic acid conjugates remained constant during germination. This information suggested that sinapine was involved in a concentration-regulated metabolism, a point of interest for plant breeders trying to lower the content of this undesirable compound in rapeseed meal (Bouchereau et al., 1992). These sinapic conjugates, however, may have a physiological function during germination and maturation stages of rape and canola seed (Shahidi and Naczk, 1995) and lowering the sinapic level may cause a deleterious effect on the early stages of development (Bouchereau et al., 1992).

Sinapic acid was shown to readily convert to a lignan, thomasidioic acid, under pH 8.5 when exposed to air (Rubino et al., 1995). Since this condition is similar to some oilseed processing conditions in industry, such as protein preparation using alkaline extraction and isoelectric precipitation, it is suspected that the same reaction could occur during oilseed processing (Rubino et al., 1995). However, the effect of thomasidioic acid on the protein quality is unknown (Rubino et al., 1995). Antioxidants such as ascorbic acid have been shown to hinder the conversion of sinapic acid to thomasidioic acid (Rubino et al, 1996a).

Dark color is one of the problems that limits the usage of canola protein as a food component (Youngs, 1991). Although the dark color of canola protein is often associated with the presence of phenolics (Shahidi and Naczk, 1992) or phenolics and phytates (Elstner and Stein, 1982), little has been done in the industry due to lack of knowledge concerning the exact role of these phenolic compounds in the systems. A study on the effect of phenolics on the color of canola protein is therefore necessary and will be of benefit to the industry.

This thesis addresses the color of canola meal and its resulting protein isolate and the relationship of color to meal phenolics. This color has been considered as a major drawback regarding canola meal utilization. The research elucidated the color and structural changes of canola phenolics, in particular, sinapine and sinapic acid, during processing. The experiments started with the structural changes in these two phenolics and the development of colored substances under conditions such as autoclaving (Cai et al, 1999a) and alkaline treatment (Cai et al, 1999b), which are commonly used in industry. Then the experiments were expanded to phenolic-protein isolate systems to determine the effect of the phenolics on

the color of protein isolates. Where necessary, new methodologies were developed.

The objectives of the thesis, therefore, were (1) to study the color and structural changes of sinapine and sinapic acid, the major canola phenolics, under conditions related to oilseed processing, (2) to study the effect of sinapine and sinapic acid on the color of canola protein isolate under similar conditions. In addition, methodologies were developed wherever necessary for color and phenolic determination.

The non-hypotheses of the thesis could be stated as follows: during autoclaving (alkaline treatment), (1) sinapine (sinapic acid) has no effect on the color of its aqueous solution; (2) sinapine (sinapic acid) has no effect on the color of canola protein isolate. With work in this thesis, all the non-hypotheses were proved false except for sinapine during autoclaving in the (1) non-hypothesis and both phenolics during alkaline treatment in the (2) non-hypothesis.

CHAPTER I. LITERATURE REVIEW

A. Composition of rapeseed and canola

Canola is a genetic variation of rapeseed developed by Canadian plant breeders specifically for its nutritional qualities (Canola Council of Canada, 1995). The improvements in nutritional qualities refer to several genetic modifications intended to increase the nutritional value of the oil and reduce the content of some antinutritional factors found in rapeseed. Typical antinutritional factors in rapeseed/canola generally refer to glucosinolates, phytates, and phenolic compounds (Ismond and Welsh, 1992; Shahidi and Naczk, 1992). So far, factors that have been genetically modified include the erucic acid, glucosinolate and fibre content in the seed. The erucic acid content in the oil and the glucosinolate content in meal have been reduced to less than 1% and 15 $\mu mol/g$, respectively, from original values of approximately 20-55% and 4%, respectively, in the traditional rapeseed varieties (Eskin et al., 1996; Swen, 1979). These canola varieties are sometimes called double low or double zero varieties (Eskin et al., 1996). Reduction in the level of glucosinolates is desirable in that they can be hydrolyzed to produce a variety of unpalatable, goitrogenic or toxic compounds such as oxazolidinethione and isothiocyanates (Shahidi, 1990, Fenwick et al., 1986, Owen et al., 1971). Oil with high amounts of erucic acid are also undesirable as they may be physiologically harmful (Swern, 1979). New varieties containing significantly lower amount of fibre are also available. A new cultivar was reported in which hull and crude fiber levels were significantly lower than black-seeded rapeseed (Krygier et al., 1982a; 1982b). No significant changes in phenolic contents of rapeseed/canola have been genetically made by

breeders, although several attempts have been reported (Krygier et al., 1982b, Fenwick, 1979,

Bouchereau et al., 1992).

Table 1.1 gives the compositions of rapeseed and canola showing the differences in

the erucic acid content and the glucosinolate content.

Table 1.1. Composition of rapeseed and canola

Component	Rapeseed [a]	Canola [b]
Oil, %	45	42.1
Erucic acid, % in oil	20-55 [c]	0.5
Oleic acid, % in oil	20.9 [c]	59.7
Protein, % N x 6.25	22	20.9
Carbohydrate, %	17	—
Chlorophyl, mg/kg	—	12
Phytic acid, %	2	—
Glucosinolates, %	4	< 15 μmol/g [d]
Phenolic and lignin, %	7	—
Hydrolyzed phenolic acid, % [e]	1.7	1-1.8

[a] Elstner and Stein, 1982, Ohlson and Anjou, 1979, dry basis, [b] Canadian Grain Commission, 1998, 8.5% moisture basis, [c] Swern, 1979, [d] Eskin et al., 1996, [e] Shahidi and Naczk, 1992.

The major components of rapeseed are oils, proteins and carbohydrates. They make

up over 80% of the whole seed. Other components are phytic acid, glucosinolate, phenolic

compounds and lignin, which make up about 10% of the total seed materials. In 1986 the definition of canola was amended to refer to *B. napus* and *B. rapa* lines containing less than 2% erucic acid in the oil and less than 30 μmol/g glucosinolates in the air dried oil-free meal (Eskin et al., 1996). In 1986, the low-erucic acid rapeseed oil was added to the generally recognized as safe (GRAS) list of food products in the United States.

The total amount of hydrolyzed phenolic acids in the canola was reported to be in the same range as in rapeseed (Table 1.1, Shahidi and Naczk, 1992). The phenolic and lignin content of rapeseed was reported to be around 7% of the whole seed (Ohlson and Anjou, 1979, Elstner and Stein, 1982).

B. Commercial value of canola

The world production of rapeseed/canola from 1948 to 1994 is listed in Table 1.2. Canadian canola production has been rising. The rapeseed production was only 11.2 thousand metric tons from 1948 to 1952. By 1984, the production was 3.24 million metric tons, accounting for 20% of the total world's rapeseed/canola production. Average Canadian canola production was 3.5 million metric tons per year from 1983 to 1992 with 3.8 million metric tons in 1992. In the 1992/1993 crop year, Canada produced 5.3 million metric tons of canola seed, planted on a recorded 4.13 million hectares (Eskin et al., 1996). On the other hand, the global production of vegetable oils is rapidly expanding as is the market share of canola and rapeseed. By 1993-1994, rapeseed/canola was the world's third most important oilseed crop, after soybean and cottonseed, with over 27.2 million metric tons produced on

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more than 20.7 million hectares (Eskin et al., 1996). The total world's oilseed production was 222 million metric tons for the year 1993/1994, with rapeseed/canola accounting for more than 12% of the world oilseed production (Eskin et al., 1996).

Table 1.2. World rapeseed/canola production from 1948 to 1999

Region	48-52[a]	1984[a]	93-94[a]	95-96[b]	97-98[b]
Canada	0.4%	20%	20%	19%	19%
Europe	26%	35%	25%	---	---
India	40%	18%	21%	---	---
China	28%	25%	25%	28%	29%
World (million metric tons)	2.8	15.9	27.2	34.5	33.3

[a] Eskin et al., 1996. [b] Statistics Canada, 1998.

Canola oil has been used in many edible and inedible oil products. The uses of canola oil and meal are summarized in Table 1.3. Canola has become one of the major agricultural cash crops in Canada (Shahidi and Naczk, 1992).

It should be noted that canola meal is currently used only as an animal feed and a fertilizer due to the presence of the previously mentioned antinutritional factors. There have been, however, many investigations looking at the use of canola protein for human consumption (Thompson et al, 1976; Liu et al., 1982). To utilize the meal has been an

interesting topic among researchers and it is also the topic of this thesis.

Table 1.3. Uses of canola oil and meal [a]

Oil		Meal
Edible	Inedible	
Shortening	Cosmetics	Animal feed
Margarine	Dust suppressants	Pet food
Salad oils	Lubricants	Fish food
Cooking sprays	Hydraulic fluids	Fertilizer
Mayonnaise	Biodiesel	
Sandwich spreads	Carriers for fungicides	
Coffee whitener	Herbicides and pesticides	
Creamers	Oil fabrics	
Prepared foods	Printing inks	
Cookies	Plasticizers	
Breads	Suntan oil	
Fried snack foods	Antistatic for paper	

[a] Canola Council of Canada, 1995.

C. An overview of the canola crushing process

Canola is commercially processed into a high quality edible oil and a meal to be used as an animal feed. The process is similar to those for the other oilseeds such as soybean, sunflower seed and flax seed. A typical process consists of seed cleaning, preheating, flaking, cooking, pressing, and solvent extraction. Processing capacities of modern Canadian oil extraction plants are in the ranges of 600-1200 metric tons of seed per day. The flow sheet of a typical canola crushing process is shown in Fig. 1.1.

Canola → Cleaning → Preheating → Flaking → Cooking →

→ Pressing → Hexane Extraction → Meal

Oil   Oil
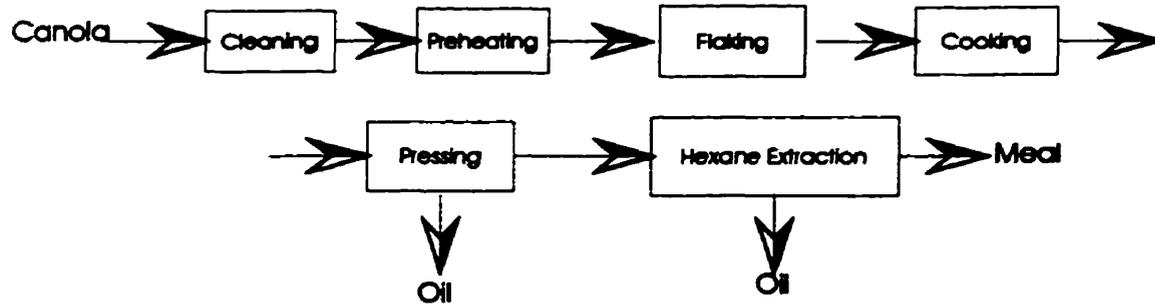
Fig. 1.1. Flow sheet of a typical canola crushing process
(Eskin et al., 1996; Shahidi, 1990).

Seed cleaning. Cleaning is to remove foreign material such as sticks, stems, leaves, and similar trash. The seed cleaning process consists of three stages: aspiration to remove dust and very light material, screening to remove oversized particles, and rescreening to remove undersized material. The equipment usually comes as one unit. The foreign material is generally reduced from approximately 6% to less than 2.5% remaining in the seed (Eskin

et al., 1996).

Preheating. Preheating is to adjust moisture content to facilitate flaking. Temperature of the seed is increased from room temperature (25 °C) to 40 °C.

Flaking. The extraction of oil from oilseeds, either by mechanical pressing or by solvent is facilitated by reduction of the seed to small particles since this reduces the distances that solvent and oil must diffuse in and out of the seed during the extraction process. In addition, flaking ruptures the cell walls, which allows some of the oil to be separated from the seed by simple pressing. The preheated seed is flaked on rolling mills. Flaking produces flakes with a thickness of 0.2-0.3 mm (Shahidi, 1990; Hamilton and Bahail, 1987).

Cooking. Cooking changes the properties of the protein so as to make the oil more easily extractable. Important secondary effects of cooking are destruction of enzymes and bacteria, and increase in the fluidity of the oil through increasing temperature (Eskin et al., 1996).

Pressing. The function of pressing is to reduce the oil content of the seed from its original level (ca. 42%) to about 16%. Extraction of the remaining oil by solvent is then much more efficient and economical. Pressure and heat is developed within the barrel by the rotating screw shaft working against an adjustable choke at the solids discharge end of the barrel (Crawford et al., 1996).

Solvent Extraction. Extraction is achieved through the principles of soaking, leaching, or washing and diffusion. The solvent is hexane, specially refined for this application. The oil content in the solid material is reduced to about 0.5%. The meal and the

miscella are stripped of the solvent to recover solvent-free meal and oil by means of steaming in a desolventizer-toster or distillation (Buhr, 1989).

D. Canola protein isolation

Several methods have been developed for producing rapeseed/canola protein isolates during the past decades. The high level of essential amino acids in rapeseed/canola and its importance as an oilseed crop in Canada and around the world has triggered interest in preparing a food grade protein isolate since the late 1960's (Eapen et al., 1969; Sosulski and Bakal, 1969; Owen et al., 1971; Liu et al., 1982).

Alkaline extraction with acidic precipitation method. A protein isolate is often prepared by first dispersing and extracting protein from the raw material with an alkaline solution. After removal of insoluble material, the dissolved proteins are recovered by acidic precipitation (Girault, 1973; Gillberg and Tornell, 1976a). The precipitate, which is usually washed and dried before use, constitutes the protein isolate. Fig. 1.2 shows a typical alkaline extraction with acidic precipitation process.
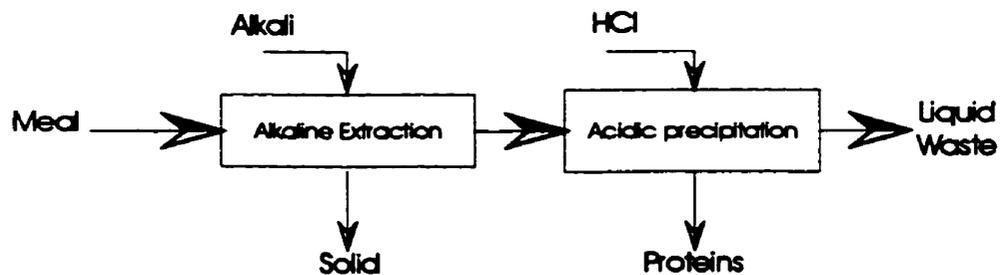
Fig. 1.2. Alkaline extraction with acidic precipitation (Gillberg and Tornell, 1976a).

While a process of this type has been successfully used on other protein isolation such as soybean, it gives low yields and produces a discarded supernatant with high protein content during rapeseed/canola protein isolation, probably due to the complicated protein composition with widely different isoelectric points and molecular weights (Gillberg and Tornell, 1976a). Anionic polysaccharides have been used as precipitants during isolation in order to increase the yield (Gillberg and Tornell, 1976b). Kodagoda et al. (1973) applied a three-step process using water, dilute hydrochloride and dilute sodium hydroxide to successively extract protein fractions and found the multi-step process offering a higher yield than the single step process. Keshavarz et al.(1977) used a similar multi-step process to extract rapeseed protein and found that while the yield of the basic extracted protein had the highest protein content and solubility, its dried product was brown and the darkest of all the protein fractions. The association of the dark color with the pH value of the extraction solutions has been observed by several other researchers (Thompson et al., 1976; Liu et al., 1982). Thompson et al. (1976) prepared a rapeseed protein isolate by extracting the protein at pH 7 with sodium hexametaphosphate (SHMP) and precipitating the protein-SHMP complex isoelectrically. The method produced a protein isolate with lighter color than those isolates where alkali was used (Thompson et al., 1976; Liu et al., 1982). In addition, isolates with a neutral final pH (pH 7) were darker than those at the isoelectric point (pH 2.5) (Liu et al., 1982).

Aqueous NaCl extraction with acidic precipitation method. In contrast to the commonly used alkaline extraction with acidic precipitation method, a method using aqueous NaCl extraction with acidic precipitation was developed by Owen et al. (1971), which

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produced a light tan, bland and nontoxic rapeseed protein isolate. Fig. 1.3 shows an aqueous

NaCl extraction with acidic precipitation process.
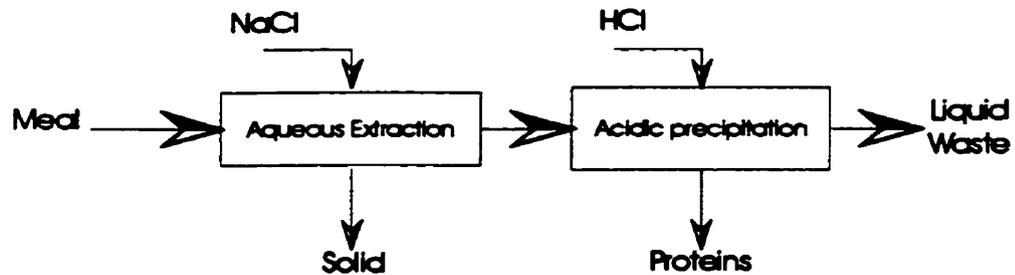
Fig. 1.3. Aqueous NaCl extraction with acidic precipitation process

(Owen et al., 1971).

In this method, the rapeseed press cake was solubilized with aqueous NaCl, a salt

solubilization step which replaced the more industrially common alkaline solubilization. After

solid matter had been removed, the salt soluble protein was precipitated isoelectrically by the

addition of hydrochloride. The well-known salting-in principle was utilized.

Aqueous NaCl extraction with precipitation by dilution method. Murray et al.

(1978) developed a novel protein isolation method using aqueous NaCl extraction with

precipitation by dilution, which eliminated the use of both alkali and acid from the process.

An aqueous NaCl extraction with precipitation by dilution process is given in Fig. 1.4.

In this method, the meal was also extracted with aqueous NaCl solution. Instead of

isoelectric precipitation, however, the proteins were precipitated by a reversal of the salting

in process, which was simply the dilution of the extract solution into cold tap water. This

resulted in the reduction in the ionic strength of the salt solubilized protein and caused a massive precipitation of the protein with the bulk of the salt remaining in the aqueous phase.
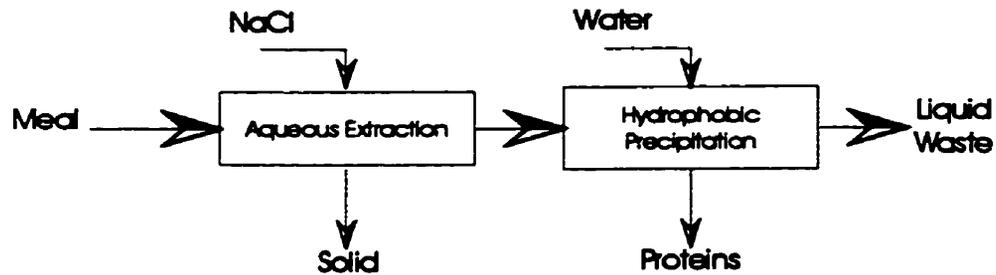
Fig. 1.4. Aqueous NaCl extraction with precipitation by dilution

(Murray et al., 1978).

The method has been well applied in fababean protein isolation and shown to be very effective in reducing antinutritional factors such as phenolics (Arntfield et al., 1985). For canola, the process was modified to include an ultrafiltration step to concentrate the salt solubilized protein and remove small molecules before the hydrophobic precipitation (Ismond and Welsh, 1992).

E. Canola protein

Canola protein mainly consists of water soluble albumins and salt soluble globulins. The globulins are the storage protein of the seed, while albumins constitute the main metabolically active proteins which are responsible for biosynthesis and degradation of globulins (Norton, 1989). The globulin fraction of canola protein contains mainly two major

fractions, the 1.7 S protein and the 12 S fraction. While the 1.7 S protein constitutes only 20% of the total protein, the 12 S protein constitutes 60% (Schwenke et al., 1983). The 1.7 S fraction has a molecular weight between 12,000 and 18,000 Dalton and an isoelectric point between 9.5 - 13, whereas the 12 S fraction has a molecular weight of about 300,000 Daltons and an isoelectric point of 7.25 (Schwenke et al., 1981). The number of subunits in the 12 S fraction has been determined to be six, each with a molecular weight of around 50,000 Daltons (Schwenke et al., 1983).

F. Discoloration caused by phenolic reactions in oilseeds and plants

Chemical reaction involving phenolic compounds or pigments. Alkaline treatment of sunflower seeds brings about the development of a distinct color that progresses from a cream yellow to light green, to dark green and eventually to brown. Thus, the conventional process for the production of protein isolates from sunflower results in dark and discolored products. This discoloration is due to the complex interaction of sunflower proteins with chlorogenic acid (Shahidi and Naczk, 1995).

During canola protein isolation, the major protein fraction extracted at pH 10 was brown in color (Keshavarz et al., 1977). Colorless powders could be obtained for all protein fractions by gassing with sulfur dioxide for 1 min after precipitation but prior to drying (Keshavarz et al.,1977; Youngs, 1991). However, this treatment may also cause the loss of essential amino acids.

In addition, anthocyanins are a type of flavonoid, and are responsible for pink, scarlet,

red, mauve, blue and violet colors of vegetables, fruits, fruit juices and wine. Anthocyanins can be bleached by sulfur dioxide. The colorless sulfonic acid derivatives are formed as a result of addition of sulfur dioxide to the 4-position of the anthocyanin molecule. The reaction is reversible (Shahidi and Naczk, 1995).

In addition to these naturally present colored compounds, there are four types of browning reactions in foods, i.e., enzymatic, Maillard, caramelization and ascorbic acid oxidation (Acton and Dawson, 1994; Eskin, 1990; Lee, 1975). Although not reported in canola, enzymatic and Maillard reactions could have occurred under appropriate conditions.

A major concern to food processors and researchers working with plant foods is the presence of polyphenoloxidase, which is mainly responsible for spoilage through enzymatic browning. This enzyme catalyzes the oxidation of phenols to highly active quinones. Subsequently, quinones may react with amino and sulfhydryl groups of proteins and with anthocyanins. These secondary reactions may bring about changes in physical, chemical and nutritional characteristics of food products. Quinones may also contribute to the formation of brown pigments due to participation in polymerization and condensation reactions with proteins (Shahidi and Naczk, 1995).

Maillard reaction is the condensation between the α-amino groups of amino acids or protein and the carbonyl groups of a reducing sugar. The condensation products, N-substituted glucosylamine, will rearrange to form fructose amino acids, which then form brown pigments through dehydration, degradation, condensation and polymerization (Eskin, 1990; Kawamura, 1983).

G. Canola phenolics

a. Occurrence and chemistry

Table 1.4 lists the contents of phenolic acids in free, esterified and insoluble-bound forms. Free and esterified phenolics are soluble in methanol, while insoluble-bound phenolics are not soluble in methanol, but released after an alkaline hydrolysis. The total contents listed in Table 1.4 are the mathematical sums of simple phenolic acids in the three forms. This is different from the total phenolic contents determined with Folin-Ciocalteu's reagent or by HPLC methods.

Table 1.4. Contents of free, esterified and insoluble-bound phenolic acids in some canola/rapeseed products

Products [a]	Phenolic acids (mg/g)			
	Free	Esterified	Insoluble-bound	Total [d]
Tower dehulled flour [b]	0.98	9.82	-	10.80
Candle dehulled flour [b]	0.85	11.96	-	12.81
Tower meal [c]	2.44	12.02	0.96	15.42
Altex meal [c]	2.48	14.58	1.01	18.07
Tower hulls [b]	0.02	1.10	0.24	1.36

[a] Tower, Altex are *B. napus* canola, Candle is *B. campestris* canola.
[b] Determined by gas chromatography (Krygier et al., 1982b). [c] (Shahidi and Naczk, 1992).
[d] Sum of phenolic acids.

The reported total content of phenolic acid in various canola protein products varies from 15.42 to 18.07 mg/g in canola meals and from 10.80 to 12.81 mg/g in dehulled flours. Hulls of Tower variety of canola contained a total phenolic content of around 1.36 mg/g.

The phenolic acids in free, esterified and bound forms of Tower canola are given in Table 1.5. The free and esterified forms were determined in the Tower flour and the insoluble-bound form was evaluated in the Tower hulls. Sinapic acid was the predominant phenolic acid in both free (90.8%) and esterified (98.6%) forms in canola flour, whereas protocatechuic acid was the predominant phenolic acid in Tower hulls. However, the contribution of phenolics from hulls is relatively small since the weight of hulls is only 6-8% of that of the whole seed.

Free phenolic acids contribute from 6.5 to 9.0% of the total phenolic acids present in most canola flours, with sinapic acid being the predominant free acid. Several other phenolic acids are present in small amounts. These include p-hydroxybenzoic, vanillic (4-hydroxy-3-methoxybenzoic), protocatechuic (3,4-dihydroxybenzoic), syringic (4-hydroxy-3,5-dimethoxybenzoic), p-coumaric (3-(4-hydroxyphenyl)-2-propenoic), cis- and trans-ferulic (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic) and caffeic (3-(3,4-dihydroxyphenyl)-2-propenoic) acids. The structures of some basic phenolic acids are given in Fig.1.5.

Table 1.5. Phenolic acids in free and esterified forms of Tower dehulled flour and insoluble bound form of Tower hulls [a]

| Phenolic acid | Phenolic acid content (mg/g) | | |
| | Dehulled Tower flour | | Tower hull |
	Free	Esterified	Insoluble-bound
p-hydroxybenzoic	trace	0.01	0.002
vanillic	0.01	trace	0.01
protocatechuic	0.003	0.002	0.16
syringic	0.02	trace	0
p-coumaric	0.03	0.02	trace
cis-ferulic	0.01	trace	trace
trans-ferulic	0.03	0.09	trace
caffeic	trace	0.01	0.04
cis-sinapic	0.09	0.74	0
trans-sinapic	0.80	8.95	0.02
Total	0.98	9.82	0.25
Sinapic acid as % of total	90.8	98.6	8

[a] Determined by gas chromatography (Krygier et al., 1982b), Tower is a B. napus canola.

Esterified phenolic acids form 91.0 to 93.5% of the phenolic acid present in canola flour (Krygier et al., 1982b). Sinapine is the principle phenolic acid ester in canola/rapeseed. The contents of sinapine in rapeseed were reported to vary from 6.0 to 12.0 mg/g (Austin and Wolff, 1968; Fenwick, 1979). A higher content of sinapine was found in B. napus rapeseed cultivars (16.5 - 22.6 mg/g) than in B. campestris cultivars (12.2 - 15.4 mg/g) (Mueller et al.,

Benzoic acid derivatives
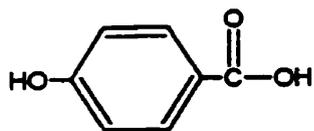
p-Hydroxybenzoic acid
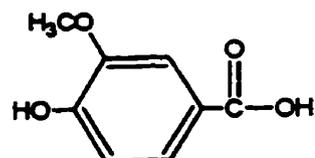
Vanillic acid
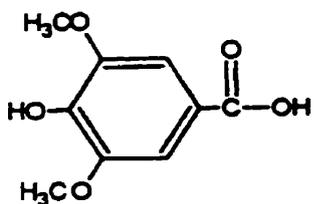
Syringic acid
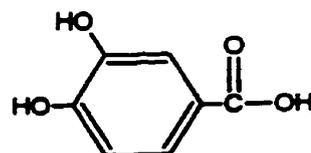
Protocatechuic
acid

Cinnamic acid derivatives
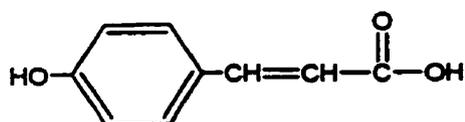
p-Coumaric acid
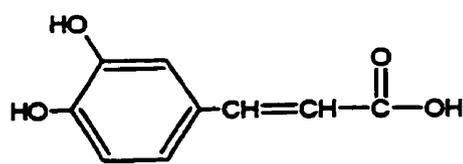
Caffeic acid
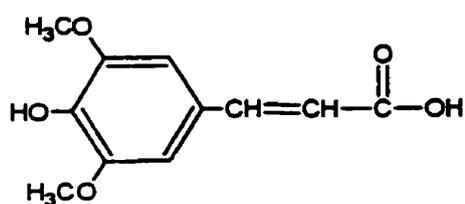
Sinapic acid
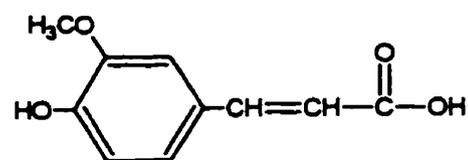
Ferulic acid

Fig. 1.5. Structures of some basic phenolic acids found in oilseeds
(Shahidi and Naczk, 1995).

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1978). At least seven other compounds yielding sinapic acid upon hydrolysis were isolated from rapeseed meals of Midas and Echo varieties (Fenton et al., 1980).

Rapeseed flours contained from 0.032 to 0.05 mg/g of insoluble-bound phenolic acids in Yellow Sarson and Gorczanski rapeseed varieties (Shahidi and Naczk, 1992). However, other sources reported that no detectable amount of bound phenolics was found in dehulled Yellow Sarson, Candle and Tower flours, while Tower hulls contained 0.25 mg/g of bound phenolics (Krygier et al., 1982b; Table 1.5).

Tannins are a group of polyphenolic compounds found in plants. They have molecular weights of 500 to 3000 Daltons (Riberean-Gayon, 1972). The reported data on tannin content varies from 0.23 to 3.91% in canola meal, and from 0.02 to 1.5% in canola hulls. Fenwick et al. (1986) reported that whole and dehulled Tower meals contained 2.71% and 3.91% tannins, respectively. On the other hand, tannin contents were reported to be 0.23 - 0.5% in defatted canola cotyledons (Shahidi and Naczk, 1992). Rapeseed hulls have been reported to contain 0.02 - 0.22% of extractable tannins (Blair and Reichert, 1984). The discrepancies in reported data are probably due to the different techniques employed in extraction and determination of tannins. Leucocyanidin was the basic unit of tannins isolated from rapeseed hulls (Shahidi and Naczk, 1992). Other units found were pelargonidin, cyanidin and its n-butyl derivative. Fig. 1.6 shows the units of condensed tannins found in oilseeds and the structure of condensed tannins found in fruit.

A
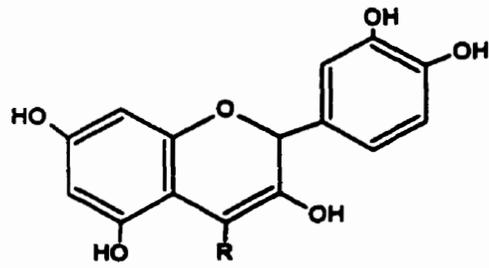
R = H: Catechin
R = OH: Leucocyanidin
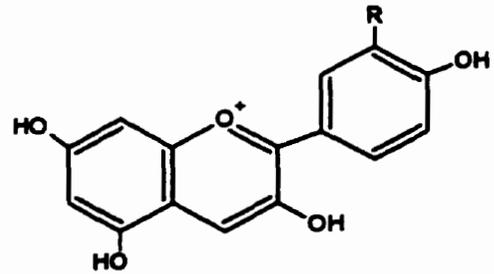
R = H: Pelargonidin
R = OH: Cyanidin

B
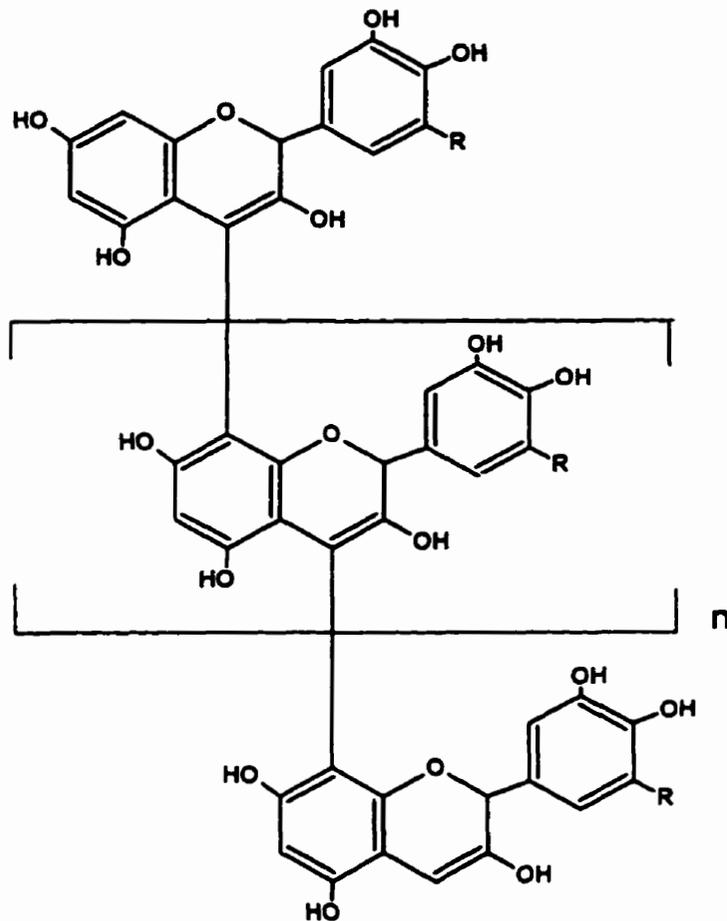
R = H: Procyanidin units, R = OH: Prodelphinidin units

Fig.1.6. Units of condensed tannins found in oilseeds (A) and the structure of condensed tannin found in fruit (B) (Shahidi and Naczk, 1995).

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b. Sinapic acid and sinapine

Sinapic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, constitutes 70.2 - 85.4% of the free phenolic acids, 70.9 - 96.4% esterified, and 7.4 - 32.1% insoluble-bound phenolics of defatted meals, respectively (Shahidi and Naczk, 1995). Sinapic acid is readily converted to thomasidioic acid under alkaline condition and in the presence of oxygen (Rubino et al., 1995). Thomasidioic acid, however, will further convert to 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid at pH 13 (Charlton and Lee, 1998). These reactions are shown in Fig.1.7.

Sinapine, the choline ester of sinapic acid, is the predominant phenolic ester in canola/rapeseed (Shahidi and Naczk, 1992; 1995). The hydrolysis of sinapine produces sinapic acid and choline (Austin and Wolff, 1968), as shown in Fig. 1.8. *Brassica napus* cultivars of rapeseed contained higher levels of sinapine than *Brassica campestris* cultivars (Mueller et al., 1978). Krygier et al. (1982b) reported that Tower (*B. napus* canola) and Candle (*B. campestris* canola) flours contained high levels of esterified phenolics ranging from 9.82 to 11.96 mg/g, respectively. It was reported that Tower hulls contained 1.10 mg/g soluble phenolic esters (Krygier et al., 1982b).

The content of sinapine was found to decrease during processing, especially heating. This was generally followed by an increase in the lignan level. The lignans occur as native rape constituents (Jensen et al., 1990).
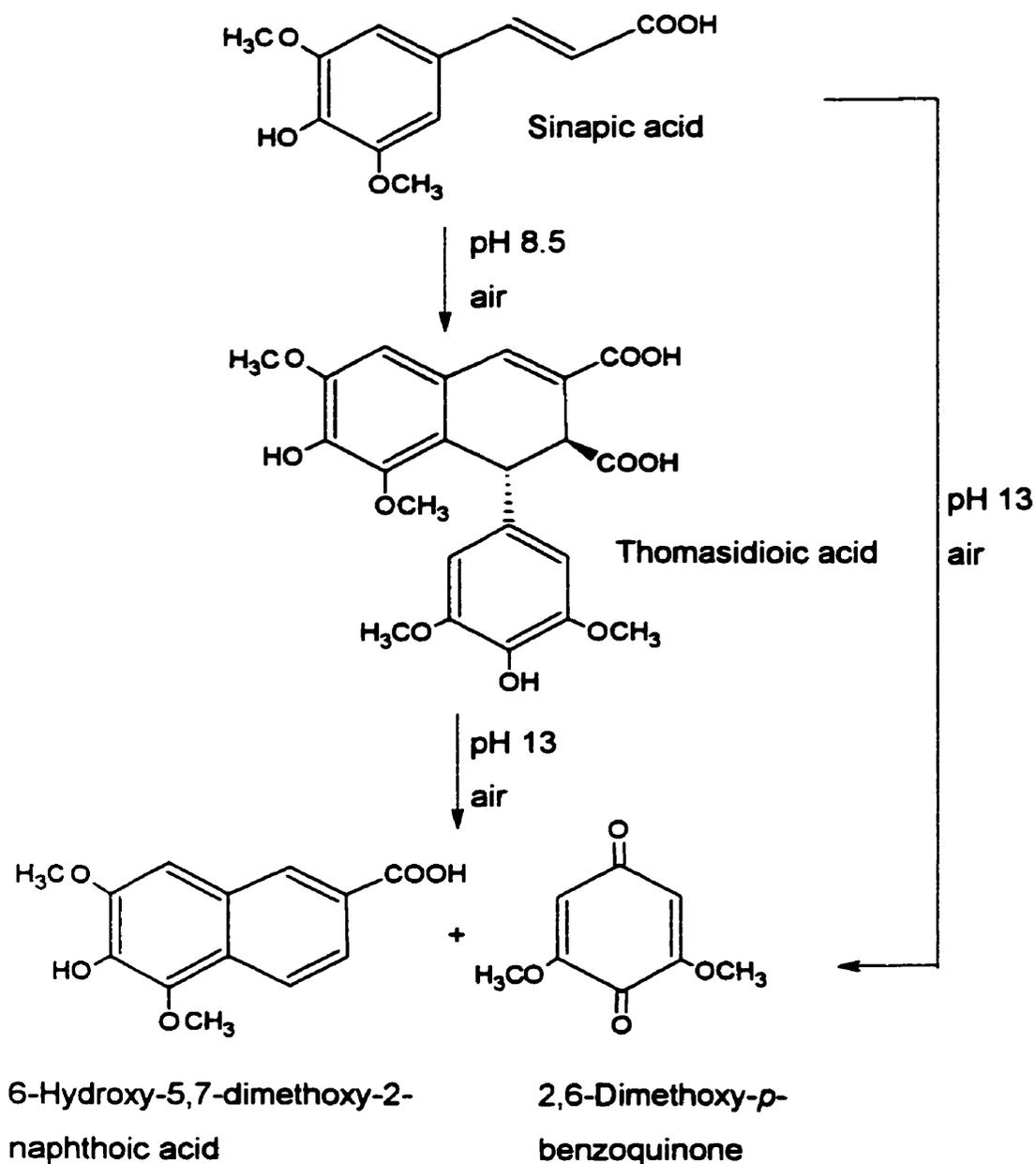
Fig. 1.7. Conversion of sinapic acid to thomasidioic acid and further to 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid (Charlton and Lee, 1997).

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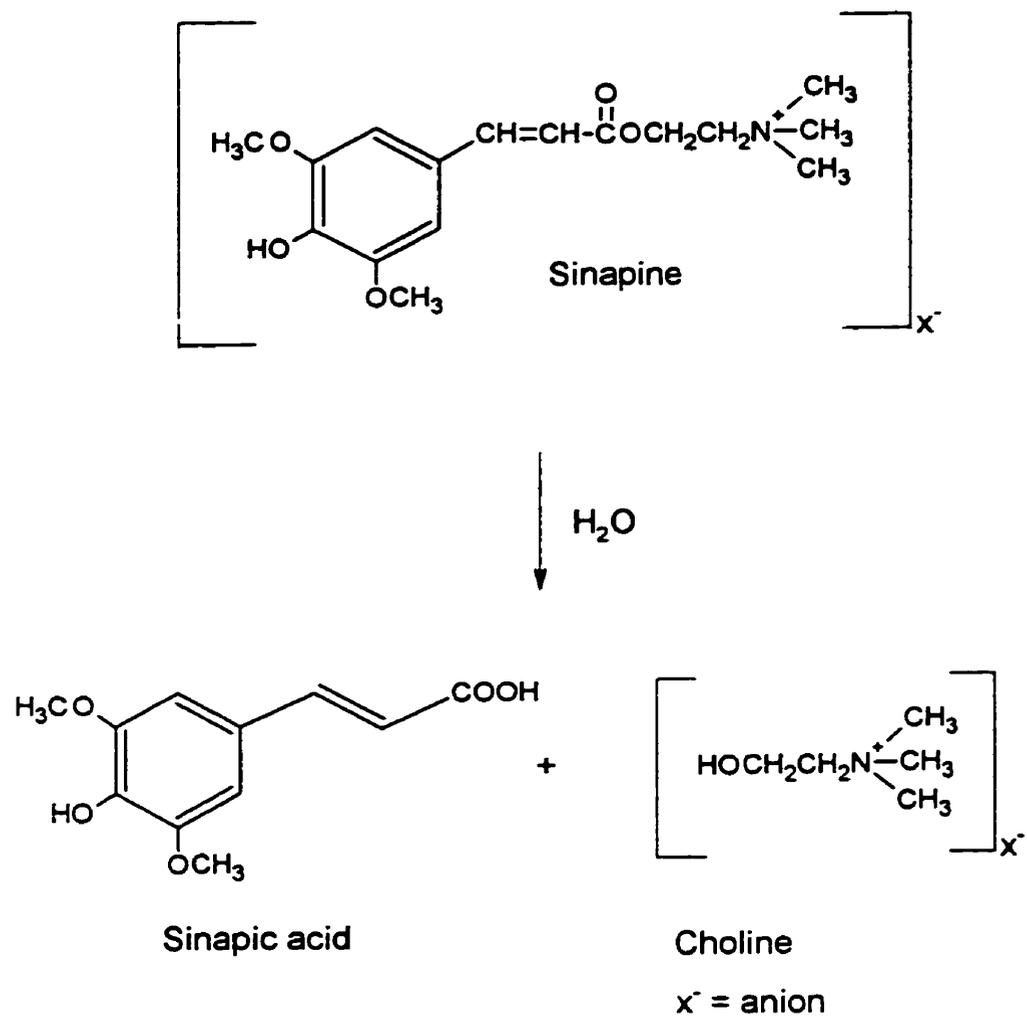
Fig. 1.8. Hydrolysis of sinapine to form sinapic acid and choline (Austin and Wolff, 1968).

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H. Determination of oilseed and plant phenolics

a. Spectrophotometric Methods

1. UV spectral characteristics of main classes of phenolics

Absorbance maxima. All phenolic substances exhibit intense absorption in the UV region of the spectrum and those that are colored absorb strongly in the visible region as well. The approximate positions of the absorption maxima of some classes of phenolics are listed in Table 1.6.

Table 1.6. Spectral characteristics of some phenolics [a]

Class	Principal maxima (nm)	Subsidiary maxima (nm)
Phenols	265 - 275	–
Cinnamic acids	230 -240	–
	310 - 350	
Coumarins	220 - 230	ca. 260 (30%)
	310 - 350	ca. 300 (30%)
Flavonols	250 - 270	ca. 300 (40%)
	350 - 390	
Anthocyanins	475 -560	ca. 275 (55%)

[a] Harborne (1964). Value in parenthesis indicates the intensity of subsidiary max. relative to that of the principal max.

Phenols have a principal maximum in the region of 265 - 275 nm. Cinnamic acids and coumarins do not differ greatly in their UV spectral characteristics, and indeed they are very similar in structure. Flavones are very distinctive, since they exhibit absorbance bands of approximately the same intensity at 250 and at 350 nm. Anthocyanins exhibit absorbance in the visible region.

Ionization of phenolics. The absorption spectra of simple phenolics have been measured in alkaline solution by several researchers (Harborne, 1964). Large bathochromic shifts from neutral or acid solutions were obtained in most cases, and an increase in intensity of the absorption bands was also observed. In alkaline solution, phenol ionizes as shown in Fig. 1.9.
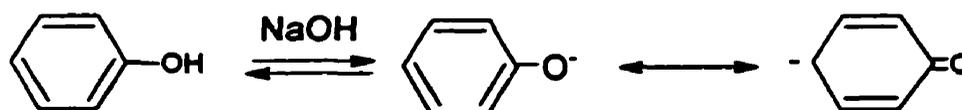
Fig. 1.9. Ionization of phenol (Harborne, 1964).

The alkaline shifts of some simple phenolics are listed in Table 1.7. Ionization of aromatic carboxylic acids causes a hypochromic shift in the spectrum (e.g., cinnamic acid, $\lambda_{max, EtOH}$ 273 nm; $\lambda_{max, alkaline}$ 267) so that phenolics containing carboxylic acid substituents do not give as large a bathochromic shift in alkali as the related esters (Harborne, 1964). This

fact is useful for distinguishing between phenolics and their esters. For example, compare sinapic acid ($\Delta\lambda$ = 48) with its choline ester, sinapine ($\Delta\lambda$ = 60) (Table 1.7).

Table 1.7. Alkaline shifts in λ_{max} of some simple phenolics

Compounds	λ_{max} neutral (nm)	λ_{max} alkaline (nm)	$\Delta\lambda$
Phenol [a]	273	291	18 (39%)
p-hydroxybenzoic acid [a]	254	280	26 —
p-coumaric acid [a]	310	335	25 —
Sinapic acid [b]	306	354	48 —
Thomasidioic acid [b]	310	354	46 (20%)
2-Naphthoic acid [b]	292	334	62 (73%)
Sinapine [b]	324	384	60 (31%)

[a] Harbome, 1964, [b] Cai et al., 1999b, alkaline = pH 10. Value in parenthesis indicates the percentage increase in the intensity of the principal max.

Direct spectroscopic determination of phenolic content. Several researchers have determined the concentration of total phenolics by measurement of the absorbance of the solution at a single wavelength, usually at the maximum nearest to or in the visible region (Swain and Goldstein, 1964).

Wang et al. (1998) used an ion-exchange UV spectrophotometric method for the determination of sinapine in Brassica seeds and meals. Phenolic extracts were prepared by a

single extraction followed by an ion-exchange column purification procedure. The sinapine content of the purified fraction was then determined using a UV spectrophotometer at 330 nm. Sinapine bisulfate was used as a standard for calibration in the test.

It should be noted that the λ_{max} and the molar absorbance for any compound are markedly affected by pH, ionic strength and the presence of certain metals and cations, as are the solvent used and the temperature at which the measurements are made (Swain and Goldstein, 1964). The change from ethanol to water as a solvent for benzoic and cinnamic acid induces ionization and causes a marked change in spectra.

2. The use of reagents in spectrophotometric methods

Most phenolic compounds are readily attacked by various oxidizing agents, many react with diazotised amines and electrophilic substances, forming colored compounds, and some form colored complexes with certain metals. These reactions are often used as the basis for estimating the level of phenolics. Since individual compounds vary widely in their ability to react with (a) oxidizing agents such as phosphomolybdate or permanganate and (b) coupling reagents such as titanium chloride, all spectrophotometric procedures for the estimation of total phenolics are necessarily empirical (Shahidi and Naczk, 1995). Table 1.8 lists some common phenolic reagents.

Folin-Denis reagent and Folin-Ciocalteu reagent methods. The Folin-Denis method is the most widely used assay for quantification of total phenolics in plants (Swain and Hillis, 1959; Shahidi and Naczk, 1995). This test is based on the reduction of

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Table 1.8. List of phenolic reagents

Reagent	Formula	Compound Formed
Folin-Denis or Folin-Ciocalteu reagent (Swain and Goldstein, 1964)	Mixture of complex phosphotungstic and phosphomolybdic acid, $H_3PW_{12}O_{40}$ and $H_3PMo_{12}O_{40}$	Oxidizes phenols and is reduced to a mixture of tungsten and molybdenum blues, W_8O_{23} and Mo_8O_{23}, broad λ_{max} 725-750 nm.
Anthocyanin reagent (Swain and Hillis, 1959)	1 mL of 30% hydrogen peroxide in 9 mL of methanolic hydrochloric acid (5/1, v/v, 3N), H_2O_2	Anthocyanin in acidic media forms red colored oxonium-carbonium ion. λ_{max} 525 nm.
Leuco-anthocyanin reagent (Swain and Hillis, 1959)	25 mL 36% (w/w) hydrochloric acid in 475 mL n-butanol	Leucoanthocyanins are transformed to anthocyanin by heating in an acidic solution, λ_{max} 550 nm.
Vanillin reagent (Swain and Goldstein, 1964; Shahidi and Naczk, 1995)		In the presence of strong acid (70% aq. H_2SO_4, v/v) gives adduct with undeactivated 1,3- and 1,3,5-hydroxyl substituted rings only. λ_{max} 500-520 nm.
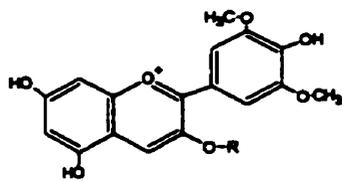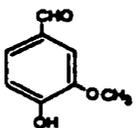
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Reinecke salt (Austin and Wolff, 1968)	Ammonium tetrathiocyano-diammonochromate	Formation of water-insoluble complex between Reinecke salt and the quaternary nitrogen base ($-NH_3^+$), dissolved in methanol for measurement, λ_{max} 400 nm.
Titanium tetrachloride (Eskin, et al., 1978; Ismail and Eskin, 1979)	$TiCl_4$	Forms complex with phenolics. broad λ_{max} 390-500 nm.
Prussion blue assay (Price and Butler, 1977)	Potassium ferricyanide	Reduction of ferric to ferrous ion (by tannin, depends on degree of polymerization), $F^{+++} \rightarrow F^{++}$, λ_{max} 720 nm.
Gibbs reagent (King et al., 1957)	2,6-Dibromoquinone chlorimide	In the presence of alkaline buffer gives indophenol. λ_{max} 620 nm.
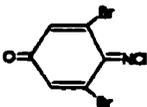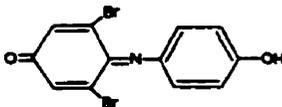
phosphomolybdic-phosphotungstic acid (Folin-Denis) reagent to a blue colored complex in alkaline solution by phenolic compounds.

In the Folin-Ciocalteu's reagent method, the Folin-Ciocalteu reagent was used instead of the Folin-Denis reagent. The procedures using Folin-Denis and Folin-Ciocalteu's reagents are similar, both involving the reduction of the reagents in saturated sodium carbonate solution. Tannic acid (Schanderl, 1970; Pearson, 1971) and ferulic acid (Velioglu et al., 1998) have been used as standards for these assays.

Anthocyanin reagent. The qualitative estimation of anthocyanins in aqueous buffer extracts of fruit tissues can be satisfactorily carried out by measuring the difference in the red color of the extract at pH 2.0 and pH 3.4 (Swain and Hillis, 1959). The change in absorbance in the visible spectrum of the anthocyanin solution with pH, is dependent on the change in the proportion of the compound in the oxonium-carbonium ion form. Anthocyanins in acidic media exist as an equilibrium between the colored oxonium ion and the colorless pseudobase form. Quantification of anthocyanins takes advantage of their characteristic behavior in the acidic media (Shahidi, and Naczk, 1995).

Leucoanthocyanin reagent. The leucoanthocyanin reagent is composed of concentrated hydrochloric acid (25 mL of 36% w/w) diluted with n-butanol to 500 mL (Swain and Hillis, 1959). All quantitative estimations of leucoanthocyanins are based on the transformation of these substances to anthocyanins by heating in an acidic solution (Shahidi and Naczk, 1995). The transformation is not 100% quantitative since a large amount of brown polymer is formed along with the anthocyanidin.

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Vanillin reagent. Vanillin reagent is composed of recrystallized vanillin (1 g) dissolved in 100 mL of 70% (v/v) concentrated sulfuric acid. The vanillin method is widely used for quantification of proanthocyanins (condensed tannins) in fruits. This method is generally recognized as a useful method for the detection and quantification of condensed tannins in plant materials due to its sensitivity, specificity, and simplicity. However, the possibility of interference with dihydrochalcones and anthocyanins should be considered (Shahidi and Naczk, 1995). This method is based on the condensation of vanillin with proanthocyanins in acidic solutions. Protonated vanillin, a weak electrophile, reacts with the flavonoid ring at the 6 and 8 positions. The intermediate product of this reaction dehydrates readily to give a light-pink to deep-red cherry colored product. The color stability of vanillin-tannin adducts may increase when light is excluded and the temperature of the reaction is controlled (Shahidi and Naczk, 1995).

Reinecke salt assay. Reinecke salt is composed of ammonium tetrathiocyano-diammonochromate (Austin and Wolff, 1968; Ismail and Eskin, 1979). This test is based on the formation of water-insoluble complex between Reinecke salt and the quaternary nitrogen base ($-NH_3^+$) such as that in sinapine. It has been used to determine sinapine (Austin and Wolff, 1968).

Titanium tetrachloride. Titanium tetrachloride reagent is composed of 20% $TiCl_4$ in concentrated HCl (Eskin et al., 1978). This method has been used to determine sinapine in rapeseed /canola and chlorogenic acid in sunflower seed (Eskin et al., 1978; Ismail and Eskin, 1979). The test is based the formation of colored complex between phenolics and

titanium tetrachloride. Aqueous ethanol or acetone was used for the extraction of phenolics from oilseed or meal.

Prussian blue assay. In this method, a reduction of ferric to ferrous ion complex is involved (Price and Butler, 1977). The ability of phenolic compounds to reduce ferric ion depends on the hydroxylation pattern and the degree of polymerization (Price and Butler, 1977). This method has been used to determine tannin content of sorghum grain.

Gibbs reagent. Gibbs reagent is selective in so far as a position para to a hydroxyl group on the phenolic ring must be free (King et al., 1957). Phenolics with substituted groups on the para position will not react with Gibbs reagent.

b. Chromatography

1. Paper and thin-layer chromatography

Thin layer and paper chromatography are widely used for purification and isolation of food phenolics (Thaller, 1964). Sosulski and Dabrowski (1984) used thin-layer chromatography to fractionate phenolics in different legume species such as lentils. The phenolic extracts from lentils were loaded on TLC plates coated with silica gel IB2-F containing fluorescent indicator UV-254. The solvent system of butanol-acetic acid-water was used for separation. The extracts from lentils fractionated by TLC indicated the presence of several colored constituents. Krygier et al. (1982a) used thin-layer chromatography to separate rapeseed phenolics. The TLC plates were coated with silica gel containing fluorescent indicator (Sosulski and Dabrowski, 1984). The solvent system employed for

separation of phenolic acid from fatty acids and other contaminants was benzene-methanol-acetic acid mixture (20:4:1). All compounds were visible on TLC plates as dark spots under short wave (254 nm) UV light while only the phenolics appeared as blue fluorescent spots under long-wave (360 nm) UV light. All acids appeared as yellow spots after spraying the plates with a 0.02% ethanol solution of bromocresol green (Krygier et al., 1982a).

In an investigation of the metabolic pathway of sinapine in rapeseed, sinapic acid derivatives were separated and characterized by thin layer chromatography on silica gel or microcrystalline cellulose plates with solvent containing n-butanol/acetic acid/water (12/3/5, v/v/v), and by paper chromatography on Whatman No 3 in a solvent containing toluene/acetic acid /water (2/1/water saturated, v/v/v) (Bouchereau et al., 1992).

2. Gas liquid chromatography (GLC)

The phenolic composition of rapeseed flour was determined by GLC (Krygier et al., 1982a, 1982b). Phenolic acids were silylated by slight warming with Tri-Sil/BSA Formula D. The Me_3Si derivatives of the phenolic acids were separated on a Hewlett-Packard Model 5710A/30A gas chromatograph equipped with a flame ionization detector and peak area integrator. The 2000x2 mm I.D. glass column was packed with 3% OV-1 on 80-100 mesh Chromosorb W (HP). The flow rate of the carrier gas was 40 cm^3/min of nitrogen. The injection and detection temperature was 300 °C. The oven temperature was programmed to run from 120 to 300 °C at 4 °C/min. Phenolic acids were identified by comparing the retention times of the Me_3Si derivatives of the unknown with those of the Me_3Si derivatives of the

standard acids (Krygier et al., 1982a).

Hartley and Jones (1975) used gas chromatography to determine the *cis* and *trans* isomers of cinnamic acids. The trimethylsilyl ether derivatives of the four substituted cinnamic acids were separated using a Pye Unicam Series 104 chromatograph with a flame ionization detector and a 2.75-m glass column (I.D. 4 mm).

3. High Performance Liquid chromatography

High-performance liquid chromatography (HPLC), with its high speed and sensitivity, offers a promising system for analyzing plant phenolics. Andersen (1983) developed a reverse- phase HPLC method for the simultaneous qualitative and quantitative determination of aglycone plant phenolic acids by using gradient elution. Phenolics were extracted from the cells with boiling ethanol, purified with centrifugation and ion-exchange separation. The purified aglycone fraction was hydrolyzed with hydrochloric acid and then extracted with diethyl ether to yield phenolic acids. These phenolic acids including gallic, protochatechuic, 4-hydroxybenzoic, vanillic, chlorogenic, syringic, ferulic, sinapic and cinnamic acids were determined with HPLC in 25 min (Andersen, 1983).

Using a similar reverse-phase HPLC method, structural changes in sinapic acid conjugates during seedling development of rape were assessed (Bouchereau et al., 1991; 1992). The seed or seedling extracts were separated into a cation fraction containing sinapine and a non-cation fraction (anions and neutral fraction) containing sinapic acid, sinapoylglucose, sinapoylmalate and 1,2-disinapoylglucose (Bouchereau et al., 1992). The

two fractions were then applied to the HPLC column. The absorbance was determined at 254 nm for sinapine and at 330 nm for the components in the non-cation fraction (Bouchereau et al., 1992).

HPLC was also used for determining phenolics in Maize mesocotyl following extraction, basic hydrolysis and purification (Hagerman and Nicholson, 1982). The reverse-phase column contained 10-μm particles of Lichrosorb C-8 and phenolics were detected at 254 nm (Hagerman and Nicholson, 1982). Cinnamic, o-coumaric, p-coumaric, caffeic, ferulic and sinapic acids were separated in 30 min with two isocratic flows at 15% and 30% methanol in sodium acetate buffer (Hagerman and Nicholson, 1982).

HPLC was also applied for the analysis of phenolics in soybean (How and Morr, 1982), grape must (Pompei et al., 1986) and eggplant (Lattanzio, 1982).

I. Color determination

a. CIE method of color specification

The CIE method is a method of color control well established in industrial practice. The letters CIE stand for Commission Internationale de l'Eclairage (International Commission for Illumination) (Acton and Dawson, 1994; Judd and Wyszecki, 1975). This international organization recommends basic standards and procedures for all aspects of light, lighting, and illuminating engineering, which includes colorimetry, the measurement of color.

b. CIE 1931 standard colorimetric observer

It has been found worthwhile to use a specific test method for color in business, industry or in science. Comparison to a standard allows for the interpretation of the spectrophotometric results. CIE recommends that colorimetric specifications of color stimuli be based on the spectral tristimulus values $x(\lambda)$, $y(\lambda)$, $z(\lambda)$, given in Table 1.9, whenever correlation with visual color matching of fields of angular subtense between 1 and 4 degrees at the eye of the observer is desired. Whenever the correlation with visual color matching of fields is of large angular subtense, that is more than 4 degrees at the eye of the observer, the colorimetric specification of color stimuli is based on the CIE 1964 supplementary standard observer (Judd and Wyszecki, 1975).

Table 1.9. CIE 1931 standard colorimetric observer [a]

Wave-length λ(nm)	Tristimulus values			Wave-length λ(nm)	Tristimulus values		
	$x(\lambda)$	$y(\lambda)$	$z(\lambda)$		$x(\lambda)$	$y(\lambda)$	$z(\lambda)$
380	0.0014	0	0.0065	580	0.9163	0.87	0.0017
385	0.0022	0.0001	0.0105	585	0.9786	0.8163	0.0014
390	0.0042	0.0001	0.0201	590	1.0263	0.757	0.0011
395	0.0076	0.0002	0.0362	595	1.0567	0.6949	0.001
400	0.0143	0.0004	0.0679	600	1.0622	0.631	0.0008
405	0.0232	0.0006	0.1102	605	1.0456	0.5668	0.0006
410	0.0435	0.0012	0.2074	610	1.0026	0.503	0.0003
415	0.0776	0.0022	0.3713	615	0.9384	0.4412	0.0002
420	0.1344	0.004	0.6456	620	0.8544	0.381	0.0002
425	0.2148	0.0073	1.0391	625	0.7514	0.321	0.0001
430	0.2839	0.0116	1.3856	630	0.6424	0.265	0
435	0.3285	0.0168	1.623	635	0.5419	0.217	0
440	0.3483	0.023	1.7471	640	0.4479	0.175	0

445	0.3481	0.0298	1.7826	645	0.3608	0.1382	0
450	0.3362	0.038	1.7721	650	0.2835	0.107	0
455	0.3187	0.048	1.7441	655	0.2187	0.0816	0
460	0.2908	0.06	1.6692	660	0.1649	0.061	0
465	0.2511	0.0739	1.5281	665	0.1212	0.0446	0
470	0.1954	0.091	1.2876	670	0.0874	0.032	0
475	0.1421	0.1126	1.0419	675	0.0636	0.0232	0
480	0.0956	0.139	0.813	680	0.0468	0.017	0
485	0.058	0.1693	0.6162	685	0.0329	0.0119	0
490	0.032	0.208	0.4652	690	0.0227	0.0082	0
495	0.0147	0.2586	0.3533	695	0.0158	0.0057	0
500	0.0049	0.323	0.272	700	0.0114	0.0041	0
505	0.0024	0.4073	0.2123	705	0.0081	0.0029	0
510	0.0093	0.503	0.1582	710	0.0058	0.0021	0
515	0.0291	0.6082	0.1117	715	0.0041	0.0015	0
520	0.0633	0.71	0.0782	720	0.0029	0.001	0
525	0.1096	0.7932	0.0573	725	0.002	0.0007	0
530	0.1655	0.862	0.0422	730	0.0014	0.0005	0
535	0.2257	0.9149	0.0298	735	0.001	0.0004	0
540	0.2904	0.954	0.0203	740	0.0007	0.0002	0
545	0.3597	0.9803	0.0134	745	0.0005	0.0002	0
550	0.4334	0.995	0.0087	750	0.0003	0.0001	0
555	0.5121	1	0.0057	755	0.0002	0.0001	0
560	0.5945	0.995	0.0039	760	0.0002	0.0001	0
565	0.6784	0.9786	0.0027	765	0.0001	0	0
570	0.7621	0.952	0.0021	770	0.0001	0	0
575	0.8425	0.9154	0.0018	775	0.0001	0	0
				780	0	0	0

[a] Judd and Wyszecki, 1975

The spectral tristimulus values $x(\lambda)$, $y(\lambda)$ and $z(\lambda)$ define the CIE 1931 standard colorimetric observer. At a given wavelength λ the values of the three functions $x(\lambda)$, $y(\lambda)$, $z(\lambda)$ give the amount of X primary, the amount of Y primary, and the amount of Z primary, respectively, required to produce for the standard observer the color of the spectral stimulus of unit radiance (Judd and Wyszecki, 1975).

c. CIE 1931 tristimulus values

The CIE 1931 tristimulus values characterize the complete spectrum of a given object-color stimulus, $\varphi(\lambda)d\lambda$, which is also called the relative spectral power distribution. It is the object-color stimulus for which we wish to determine the CIE tristimulus values.

The CIE 1931 tristimulus values **X**, **Y**, **Z** of an object-color stimulus $\varphi(\lambda)d\lambda$ are defined by the following equations:

$$X = k \int_\lambda \varphi(\lambda)\, x(\lambda)\, d\lambda$$

$$Y = k \int_\lambda \varphi(\lambda)\, y(\lambda)\, d\lambda$$

$$Z = k \int_\lambda \varphi(\lambda)\, z(\lambda)\, d\lambda \quad \text{(Judd and Wyszecki, 1975, Francis, 1983)}$$

wkere k is a normalizing factor, which for object-color stimuli is conveniently chosen as

$$k = 100 / \int_\lambda S(\lambda)\, y(\lambda)\, d\lambda$$

and $\varphi(\lambda)d\lambda$ is the relative spectral power distribution which defines the object-color stimuli such that,

$$\varphi(\lambda)\, d\lambda = \tau(\lambda)\, S(\lambda)d\lambda$$

where $\tau(\lambda)$ is the spectral transmittance factor (spectral reflectance factor for opaque objects), and $S(\lambda)$ the relative spectral distribution of CIE standard illuminate D_{65} to represent average daylight.

For a self-luminous color stimulus, the equation is simply:

$$\varphi(\lambda)\, d\lambda = S(\lambda)\, d\lambda$$

where $S(\lambda)$ is the relative spectral power distribution of the self-luminous color stimulus, for example, a fluorescent lamp.

To evaluate the integrations in the above equations which give tristimulus values X, Y, Z, an approximation of the integration by a summation known as weight-ordinate method is usually taken. In this method, the spectrum is broken down into a finite number of equal wavelength intervals $\Delta\lambda$ and the CIE 1931 tristimulus values are obtained from the following sums:

$$X = k \sum \varphi(\lambda) \, x(\lambda) \, \Delta\lambda$$

$$Y = k \sum \varphi(\lambda) \, y(\lambda) \, \Delta\lambda$$

$$Z = k \sum \varphi(\lambda) \, z(\lambda) \, \Delta\lambda \qquad\qquad \text{(Judd and Wyszecki, 1975)}$$

with

$$k = 100 \, / \sum S(\lambda) \, y(\lambda) \, \Delta\lambda$$

and

$$\varphi(\lambda) \, \Delta\lambda = \tau(\lambda) \, S(\lambda) \, \Delta\lambda$$

This method of calculation is called the weight-ordinate method since each ordinate of the curve, $\varphi(\lambda) \, \Delta\lambda = \tau(\lambda) \, S(\lambda) \, \Delta\lambda$, is weighted by the CIE 1931 standard tristimulus values $x(\lambda)$, $y(\lambda)$, $z(\lambda)$. Usually, $\Delta\lambda = 5$ nm or even $\Delta\lambda = 10$ gives a sufficient approximation.

Once we have calculated the tristimulus values of a color stimulus, the chromaticity coordinates can be found from the following equations:

$$x = X/(X + Y + Z)$$

$$Y = Y/(X + Y + Z)$$

CIE 1931 tristimulus values X, Y, Z do not directly correspond to the real color intensity. They can be converted to Hunter L a b values using the following equations:

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$$L = 10 \, Y^{\frac{1}{2}}$$

$$a = 17.5 * (1.02X - Y) / Y^{\frac{1}{2}}$$

$$b = 7.0 * (Y - 0.847Z) / Y^{\frac{1}{2}} \qquad \text{(Judd and Wyszecki, 1975)}$$

Variations of lighter or darker are called lightness differences, variations of grayer or more saturated are called saturation differences, and variations toward any contiguous member of the series, red, yellow, green and blue, are called hue differences. Lightness, saturation and hue can be calculated from the Hunter L a b values as follows:

$$\text{Lightness} = L$$

$$\text{Saturation} = (a^2 + b^2)^{\frac{1}{2}}$$

$$\text{Hue} = \theta = \tan^{-1} b/a \qquad \text{(Acton and Dawson, 1994)}$$

Hunter L a b values represent the real color intensity and have been applied to color-production control tasks in various industries. A Hunter L a b color solid is given in Fig. 1.10.
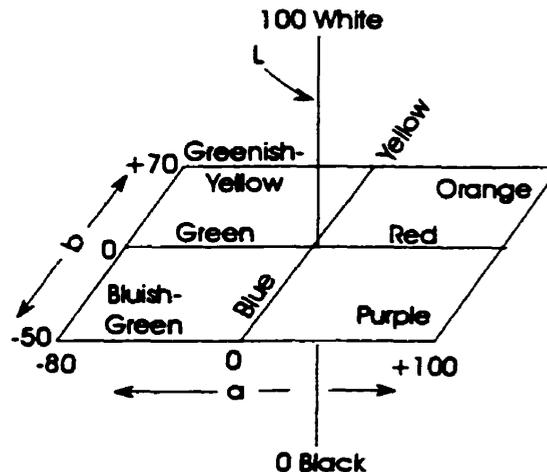
Fig. 1.10. Hunter L a b color solid (Acton and Dawson, 1994)

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Thus, while instruments are available that generate these values, it is also possible to calculate L a b values from direct spectrophotometric readings of the visible wavelengths.

Details of the calculations of the tristimulus values X, Y, Z for an object-color stimulus $\varphi(\lambda)$ $\Delta\lambda$ = $\tau(\lambda)$ S(λ) $\Delta\lambda$ are shown in Table 1.10. The object was a 100 µg/mL sinapine solution of pH 10, which had a greenish-yellow color. Its spectral transmittance factors $\tau(\lambda)$ have been measured by a spectrophotometer and are given in the second column of Table 1.10 for the wavelength range from 380 to 780 nm at wavelength intervals $\Delta\lambda$ = 10 nm. The illuminant is represented by S(λ) of the CIE standard illuminant D_{65} in the third column. The observer viewing the object is represented by the CIE 1931 standard colorimetric observer defined by the spectral tristimulus values $x(\lambda)$, $y(\lambda)$, $z(\lambda)$ given in the fourth, fifth and sixth columns of Table 1.10. The seventh through the tenth columns of Table 1.10 show the results of the first phase of the calculation which gives for each wavelength λ the products as indicated.

In the second phase the sums ($\sum_{\lambda}$) of each column of the products are formed and they are entered in the equations for k, X, Y, Z shown in the bottom of the table.

The calculated tristimulus values can then be used to derive the chromaticity coordinates x, y, Hunter L a b values and lightness, saturation and hue of the given object-color stimulus, as given in the bottom of Table 1.10.

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Table 1.10. Calculation of the tristimulus X Y Z values, Hunter L a b values, chromaticity coordinates, saturation and hue of an object-color stimulus, 100 μg/mL sinapine in a phosphate-boric acid buffer of pH 10 [a]

Wave-length λ(nm)	Object τ(λ)	D_{65} Source S(λ)	CIE 1931 standard observer			Products			
			x(λ)	y(λ)	z(λ)	τ*S*x	τ*S*y	τ*S*z	S*y
380	0	50	0.0014	0	0.0065	0	0	0	0
390	0	54.6	0.0042	0.0001	0.0201	0	0	0	0
400	0	82.8	0.0143	0.0004	0.0679	0	0	0	0
410	0	91.5	0.0435	0.0012	0.2074	0	0	0	0.1
420	0.001	93.4	0.1344	0.004	0.6456	0	0	0	0.4
430	0.001	86.7	0.2839	0.0116	1.3856	0	0	0.2	1
440	0.013	104.9	0.3483	0.023	1.7471	0.5	0	2.3	2.4
450	0.101	117	0.3362	0.038	1.7721	4	0.4	21	4.4
460	0.357	117.8	0.2908	0.06	1.6692	12.2	2.5	70.2	7.1
470	0.665	114.9	0.1954	0.091	1.2876	14.9	7	98.4	10.5
480	0.861	115.9	0.0956	0.139	0.813	9.5	13.9	81.1	16.1
490	0.942	108.8	0.032	0.208	0.4652	3.3	21.3	47.7	22.6
500	0.968	109.4	0.0049	0.323	0.272	0.5	34.2	28.8	35.3
510	0.972	107.8	0.0093	0.503	0.1582	1	52.7	16.6	54.2
520	0.973	104.8	0.0633	0.71	0.0782	6.5	72.4	8	74.4
530	0.973	107.7	0.1655	0.862	0.0422	17.3	90.3	4.4	92.8
540	0.974	104.4	0.2904	0.954	0.0203	29.5	97	2.1	99.6
550	0.975	104	0.4334	0.995	0.0087	43.9	100.9	0.9	103.5
560	0.974	100	0.5945	0.995	0.0039	57.9	96.9	0.4	99.5
570	0.973	96.3	0.7621	0.952	0.0021	71.4	89.2	0.2	91.7
580	0.988	95.8	0.9163	0.87	0.0017	86.7	82.3	0.2	83.3
590	0.979	88.7	1.0263	0.757	0.0011	89.1	65.7	0.1	67.1
600	0.977	90	1.0622	0.631	0.0008	93.4	55.5	0.1	56.8
610	0.976	89.6	1.0026	0.503	0.0003	87.7	44	0	45.1
620	0.979	87.7	0.8544	0.381	0.0002	73.3	32.7	0	33.4
630	0.981	83.8	0.6424	0.265	0	52.8	21.8	0	22.2
640	0.978	83.7	0.4479	0.175	0	36.7	14.3	0	14.6
650	0.979	80	0.2835	0.107	0	22.2	8.4	0	8.6
660	0.984	82.2	0.1649	0.061	0	13.3	4.9	0	5
670	0.976	82.3	0.0874	0.032	0	7	2.6	0	2.6
680	0.978	78.3	0.0468	0.017	0	3.6	1.3	0	1.3

690	0.978	69.7	0.0227	0.0082	0	1.5	0.6	0	0.6
700	0.98	71.6	0.0114	0.0041	0	0.8	0.3	0	0.3
710	0.978	74.3	0.0058	0.0021	0	0.4	0.2	0	0.2
720	0.979	61.6	0.0029	0.001	0	0.2	0.1	0	0.1
730	0.977	69.9	0.0014	0.0005	0	0.1	0	0	0
740	0.977	75.1	0.0007	0.0002	0	0.1	0	0	0
750	0.976	63.6	0.0003	0.0001	0	0	0	0	0
760	0.98	46.4	0.0002	0.0001	0	0	0	0	0
770	0.978	66.8	0.0001	0	0	0	0	0	0
780	0.977	63.4	0	0	0	0	0	0	0

$k = 100 / \sum S * y = 0.0946$ $\sum -$ 841.6   1013.5   382.6 1057.1

CIE 1931 tristimulus values

$X = k \sum \tau * S * x = 79.6$

$Y = k \sum \tau * S * y = 95.9$

$Z = k \sum \tau * S * z = 36.2$

$Y^{1/2} = 9.79$

CIE1931(x, y)-chromaticity coordinates

$x = X/(X + Y + Z) = 0.376$

$y = Y/(X + Y + Z) = 0.453$

Hunter L, a, b values

$L = 10 * Y^{1/2} = 97.9$

$a = 17.5 * (1.02X-Y)/Y^{1/2} = -26.2$

$b = 7.0 * (Y-0.847Z)/Y^{1/2} = 46.6$

Lightness = L

Saturation = $(a^2 + b^2)^{1/2} = 53.5$

tanb/a = 4.75

Hue = $\tan^{-1}b/a = -60.6$

[a] Cai et al., 1999a; 1999b.

CHAPTER II. STRUCTURAL CHANGES OF SINAPIC ACID AND SINAPINE BISULFATE DURING AUTOCLAVING WITH RESPECT TO THE DEVELOPMENT OF COLORED SUBSTANCES

ABSTRACT

Structural changes in sinapic acid during autoclaving were studied using spectral analysis, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and mass spectroscopy. Color properties of sinapic acid and its derivatives were studied by determining the transmittance spectrum, calculating the Commission Internationale de l'Eclairage (CIE) 1931 tristimulus values and converting to Hunter L a b values. It was found that the colorless sinapic acid aqueous solution (100 µg/mL) turned yellow after 15 minutes in an autoclave at 121 °C and 0.1 MPa. Filtering the yellow aqueous solution through a 0.45-µm filter removed a brown solid consisting of at least three undetermined colored substances and left a yellow liquid. A newly developed yellow substance, syringaldehyde was identified in the liquid phase by comparing the NMR and mass spectrum of the unknown with those of authentic syringaldehyde. Thomasidioic acid was also found in the liquid phase. Under the same autoclaving conditions, sinapine showed no evidence of any structural or color change.

KEY WORDS: Autoclaving, canola phenolics, chromatographic analysis, color, sinapic acid, sinapine, syringaldehyde.

INTRODUCTION

Canola provides an excellent edible oil source (Ackman, 1990) and is one of the most important oilseeds in the world (Eskin et al., 1996; Shahidi, 1990). Canola protein has a well-balanced amino acid content and a favorable protein efficiency ratio (Liu et al., 1982; Ohlson and Anjou, 1979). Therefore, interest in preparing food grade protein from canola meal has been increasing (Ismond and Welsh, 1992; Gillberg and Tornell, 1976a; Owen et al., 1971). Unfortunately, the use of canola protein is limited by the presence of some antinutritional compounds such as glucosinolates, phytates and phenolics (Shahidi and Naczk, 1992). Phenolics have significant effects on the visual attributes, flavor characteristics, nutritional and even functional properties of oilseed products (Shahidi and Naczk, 1992; 1995; Youngs, 1991; Rubino et al., 1996b). Color and some other effects are believed to be developed during oilseed processing (Youngs, 1991). Commercial defatting of canola is currently accomplished almost exclusively by pre-press and solvent extraction systems, with flaking and cooking of the whole seed before pressing in expellers (Eskin et al., 1996; Hamilton and Bahail, 1987; Buhr, 1989). The severe moist-heat treatment, in the cooker and desolventizer unit, darkens the meal color and denatures protein (Youngs, 1991). The resulting commercial canola meals are less than ideal for food use partly because of the dark color and bitter flavor associated with products produced from phenolics during processing. Determination of the reactions involving these phenolics that would occur during processing is the key to further research on this problem.

In contrast to the effect of pH on the color properties of oilseed phenolics (Ribereau-

Gayon, 1972; Harborne, 1964; Austin and Wolff, 1968), which generally occurs during protein isolation (Keshavarz et al., 1977), the effect of heat and pressure are closely related to the steps during oilseed extraction, such as preheating, cooking, pressing and desolventization (Eskin et al., 1996; Shahidi, 1990; Hamilton and Bahail, 1987). During these steps, heat and pressure are normally required. In a study on sunflower seed phenolics, heating was found to decrease the content of the simple phenolics (Sastry and Subramanian, 1985). Heat treatments were also shown to result in a decrease in the content of sinapine and an increase in lignan content in rapeseed (Jensen et al., 1990). Heat and pressure seemed to have significant effects on the color and structures of phenolics. However, the exact nature of these changes has rarely been reported, partly because of the complexity of the systems. In addition, there appeared to be no reports on the coloration of canola phenolics during autoclaving.

Sinapic acid and sinapine are the focus of this paper since they are the major simple and esterified phenolics in canola (Shahidi and Naczk, 1992; Blair and Reichert, 1984; Sosulski and Dabrowski, 1984; Krygier et al., 1982b; Fenton et al., 1980). The present study reports the structural changes and coloration of sinapic acid and sinapine during autoclaving with a focus on the development of colored substances. The experiments were performed with pure phenolic solutions to ensure that the information obtained reflected the substances of interest.

MATERIALS AND METHODS

A. Sources of Materials

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Syringaldehyde is a dull yellow solid (Sigma-Aldrich Canada Ltd., material safety data sheet, 1997). Aldrich silica gel (230-400 mesh, 60 Å) was used for column chromatography. Sinapine was isolated from *Sinapis alba* certified seed from Tilney Mustard crop as sinapine bisulfate according to the method outlined by Clandinin (1961). Acetic acid and sodium hydroxide used for HPLC were verified ACS- grade and purchased from Fisher Scientific Co. (Nepean, ON, Canada). Other chemicals used for HPLC were HPLC-grade. All other chemicals, unless stated otherwise, were verified ACS-grade and purchased from Fisher Scientific Co.

Thomasidioic acid was prepared according to Ahmed et al. (1973) and Rubino et al. (1995) with some modifications. Twenty mg sinapic acid were dissolved in 0.4 mL methanol. This solution was added to an aqueous $FeCl_3$ solution (40 mg/1.6 mL). The solution was aerated for a few seconds. The violet-red precipitate was collected and treated with concentrated H_2SO_4 for 30 seconds. After diluting with an equal amount of water, the resulting mixture was extracted with ethyl acetate (3x5 mL), dried with $MgSO_4$, and evaporated under vacuum. The residue was dissolved in methanol, activated carbon added, the mixture was then filtered through a 0.45-μm filter. The methanol was evaporated under

vacuum. The resulting thomasidioic acid was used as a standard for the identification of new compounds formed during autoclaving. Thomasidioic acid is a colorless solid (Ahmed et al., 1973).

B. Procedure for determination of structural changes and coloration of sinapic acid

The procedure for the determination of color and structural changes of sinapic acid is shown in Fig.2.1. A 100 μg/mL solution was prepared by dissolving 200 mg of sinapic acid in 2000 mL distilled water in a 3000-mL beaker. About 10 mL of this solution was kept as a control sample. In determining the effect of autoclaving time on the color properties of the solution, three samples of 10 mL each were taken from the above solution and autoclaved for 15, 30, and 45 min, respectively. The rest of the solution was autoclaved for 30 min. Autoclaving was conducted with an AMSCO Eagle 3000 Series Sterilizer (American Sterilizer Company, Horsham, PA). Time, temperature, and pressure were controlled automatically. A gravity model was used for the autoclaving of the samples, where no liquid water was added during autoclaving. Temperature and pressure were routinely set to 121 °C (250 F) and 0.1 MPa (15 Psi). Spectral analysis of the samples was conducted both before and after autoclaving to determine the UV spectra and Hunter L a b values. After autoclaving, the solution was filtered through a 0.45-μm filter, giving a brown solid phase and a yellow liquid phase.

Analysis of the brown solid. The solid phase was recovered from the filter paper by washing with ethyl acetate, drying with $MgSO_4$ and evaporating under vacuum to remove
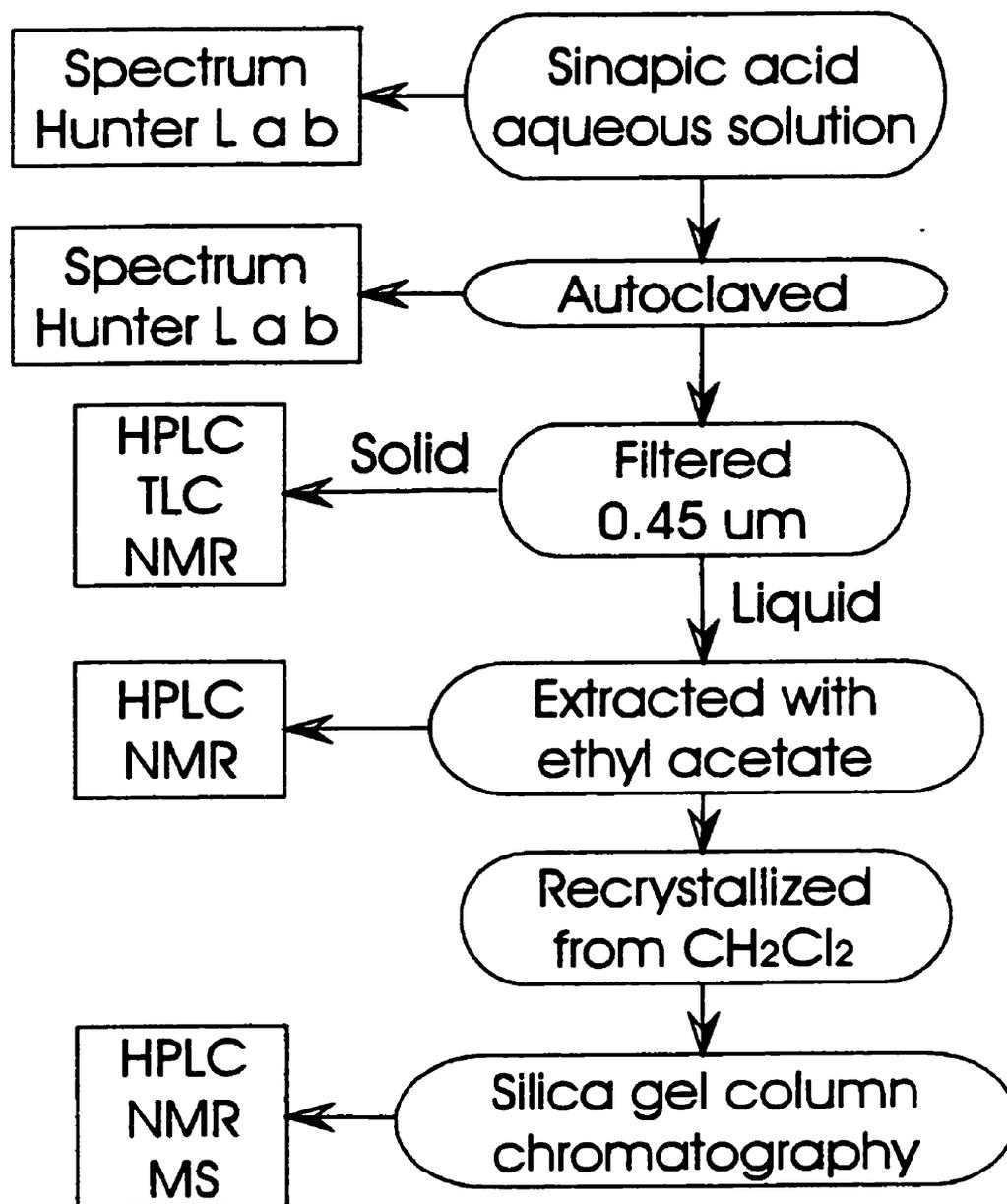
Fig. 2.1. Procedure for determining structural changes and color properties of sinapic acid during autoclaving.

solvent. HPLC analysis and thin layer chromatography (100% ethyl acetate) were conducted on the recovered solid samples. Three fractions from the thin layer chromatography at R_f 0.95, 0.91 and 0.72 were recovered from the silica gel by extracting with methanol, filtering through a 0.45-µm filter to remove silica gel and evaporating under vacuum to remove the solvent. An NMR spectrum analysis was performed for each of these fractions.

Analysis of the yellow liquid. The yellow liquid phase from the filtration was analyzed immediately following the autoclaving and filtration using HPLC analysis. Also a 10-mL sample was kept as a control for later HPLC analysis. The substances in the rest of the liquid phase were recovered by extracting the solution with ethyl acetate. The organic phases were combined, dried with $MgSO_4$ and evaporated under vacuum to remove the solvent. After recording its ^1H NMR spectrum, the residue, a red solid, was recrystallized from CH_2Cl_2. The crystals, which were mainly sinapic acid (TLC, NMR), were discarded, while the liquid CH_2Cl_2 solution was chromatographed through a 300x2-cm silica gel column using ethyl acetate as eluant. The elution was run under slight air pressure and 20 fractions were collected. These fractions were evaporated to dryness under a stream of warm air. The 4[a] fraction, a dull orange solid was the major fraction. This solid was analyzed using NMR spectroscopy, mass spectroscopy and HPLC.

C. Preparation and treatment of sinapine solution

A 100 µg/mL sinapine solution was prepared by dissolving 5 mg of sinapine into 50 mL distilled water. While 10 mL of this solution was kept as control, the rest was autoclaved

for 45 min under the same conditions as used for sinapic acid. Spectral and HPLC analysis were performed for the control and the autoclaved sample.

D. Spectral analysis and color determination

Spectral analyses were performed with a Hewlett-Packard 8452 diode array spectrophotometer with MS-DOS UV-VIS software looking at both UV (200 - 350 nm) and visual spectra (380 - 780 nm). The same samples were used for both spectral and HPLC analysis. Ultraviolet spectra were recorded as curves of absorbance as a function of wavelength. Visual spectra were recorded as percentage of light transmittance as a function of wavelength. The color of a solution was determined by taking the transmittance data at the wavelength range from 380 to 780 nm, calculating the CIE 1931 tristimulus values, X, Y and Z, and mathematically converting to Hunter L, a and b values (Judd and Wyzecki, 1975).

The calculation of the CIE 1931 tristimulus values, X, Y and Z from the above transmittance data was based on a weight-ordinate method with a wavelength interval $\Delta\lambda=10$ (28). This calculation was done with a computer spreadsheet Program (Quattro Pro, Version 7, Corel Corporation Limited, Canada). The CIE standard illuminant D_{65} was used as the illuminant in the calculation. Since these X, Y, and Z values do not have a direct proportion to real color intensities, they were converted to Hunter L a b values using the equations $L=10*Y^{0.5}$, $a= 17.5*(1.02*X-Y)/Y^{0.5}$, and $b=7.0*(Y-0.847*Z)/Y^{0.5}$ (Judd and Wyzecki, 1975).

E. HPLC Analysis

HPLC was used to monitor the structural changes in sinapic acid and sinapine following the autoclave treatment. Chromatographic equipment consisted of two Waters (Milford, MA) pumps (model 501 and 510), an automated gradient controller model 680 (Waters), a Shimadzu (Kyoto, Japan) SPD-6A ultraviolet (UV) spectrophotometric detector, and a Hewlett-Packard (Avondale, PA) model HP3396II integrator connected with a peak 96 computer software (Hewlett-Packard Company, Avondale, PA). A reverse-phase column (Supelcosyl, 3-μm particle size, 33x4.6 mm i.d.; Supelco, Bellefonte, PA) was used. The elution solvent consisted of two components. Component A was a 0.05 M sodium acetate buffer prepared by a 1:100 dilution of a stock pH 4.7 acetate buffer. The stock buffer was prepared by adjusting 5 M acetic acid to pH 4.7 with solid sodium hydroxide (Hagerman and Nicholson, 1982). This acetate was filtered through a 0.45 μm filter. Component B was pure methanol. The column was maintained at 37 °C and run at a constant flow rate of 1.4 mL/min.

For sinapic acid solutions before and after autoclaving, the initial elution solvent was 15% methanol and 85% component A. After a 10-min isocratic flow, a 2-min linear gradient was used to change the solvent composition to 100% methanol. This composition was maintained for 2 min, after which another 2-min linear gradient returned the solvent to its original composition.

For the brown solid separated by filtration through a 0.45-μm filter, the initial elution solvent was 15% methanol and 85% component A. This composition was altered to 100% methanol with a 10-min linear gradient. The solvent composition was maintained at 100%

methanol for 2 min, after which a 2-min linear gradient returned the solvent to the original composition. The brown solid was dissolved in methanol for the HPLC analysis. The same solvent system was used for sinapine solutions. Sinapine was eluted within the 10-min linear gradient. Mixtures of syringaldehyde and sinapic acid were separated by the same linear gradient solvent.

F. Thin layer chromatography (TLC)

In order to qualitatively analyze the colored components, TLC was performed using silica gel TLC plates (Waterman, Clifton, NJ), with 100% ethyl acetate as elution solvent. Crude reproductions of the results were made using a computerized drawing program (Corel Flow, version 7, Corel Corporation Limited, Canada). R_f was measured manually.

G. Nuclear magnetic resonance (NMR) and mass spectra

NMR and mass spectral analyses were performed on several materials obtained during the isolation procedure. Samples were dissolved in acetone D_6, filtered through a pipet filled with Kimwipes paper (Kimberly-Clark Corp., Roswell, GA) and collected in NMR tubes for analysis.

The ^{1}H and ^{13}C NMR spectra were recorded using a Brucker AM-300 spectrometer (Karlsruhe, Germany) with tetramethylsilane as internal standard. Acetone D_6 was used as solvent for all of the NMR analyses. Mass spectra were obtained on a vacuum Generator, model VG 7070E-HF instrument (Manchester, England).

RESULTS AND DISCUSSION

Sinapic Acid

A. Spectral and color changes during autoclaving (development of yellow appearance)

UV and visual spectral changes for sinapic acid solutions before and after autoclaving are shown in Fig.2.2 a-b. The UV spectrum of the sinapic acid solution changed from two major peaks (maximum around 230 and 320 nm) to three major peaks (maximum around 210, 225, and 310). There was an increased absorbance in the region above 380 nm after autoclaving (Fig.2.2a). It appears that about 40% of sinapic acid was converted to new substances during autoclaving, and that the UV spectrum still mainly represented sinapic acid.

The effects of autoclaving on the visual transmittance spectrum of a 100 μg/mL sinapic acid solution were more dramatic (Fig.2.2b). After autoclaving, the solution exhibited a decreased percentage transmittance (increased absorbance), especially in the range from 380 to 480 nm. This would be consistent with the yellow appearance of the solution, since the maximum absorbance occurred in the blue wavelength region. A more precise determination of color was done by calculating the CIE 1931 tristimulus values, X, Y, and Z and converting to the Hunter L a b values.

The effect of autoclaving time on the Hunter L a b values of a 100 μg/mL sinapic acid solution is shown in Fig.2.3. Autoclaving treatment decreased whiteness (L value), while significantly increasing yellow (b value) and slightly increasing green color. As a result, the appearance of the solution turned from colorless to yellow after autoclaving. Increasing the
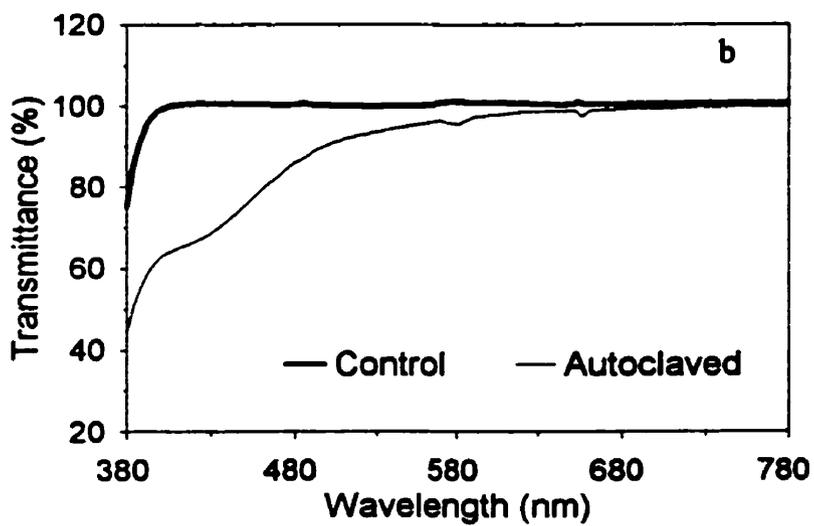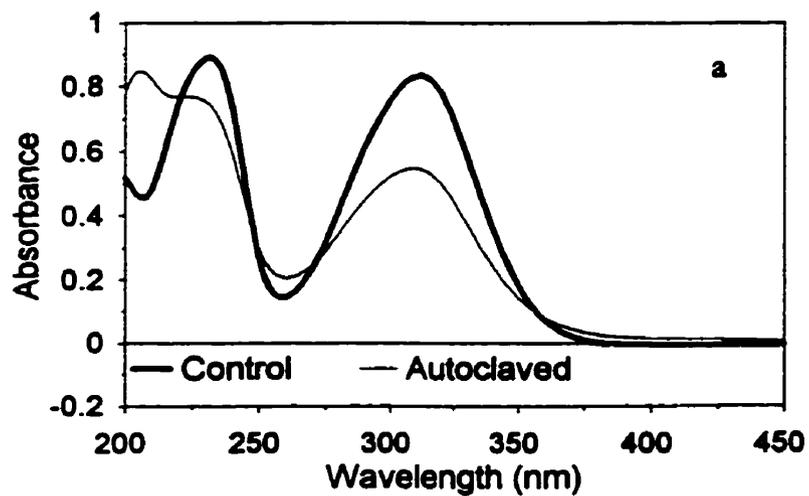
Fig. 2.2a-b. Effect of autoclaving (121 °C, 0.1 MPa, 15 min) on the UV spectrum (diluted 10 times, a) and visual transmittance spectrum (b) of a 100 μg/mL sinapic acid solution.
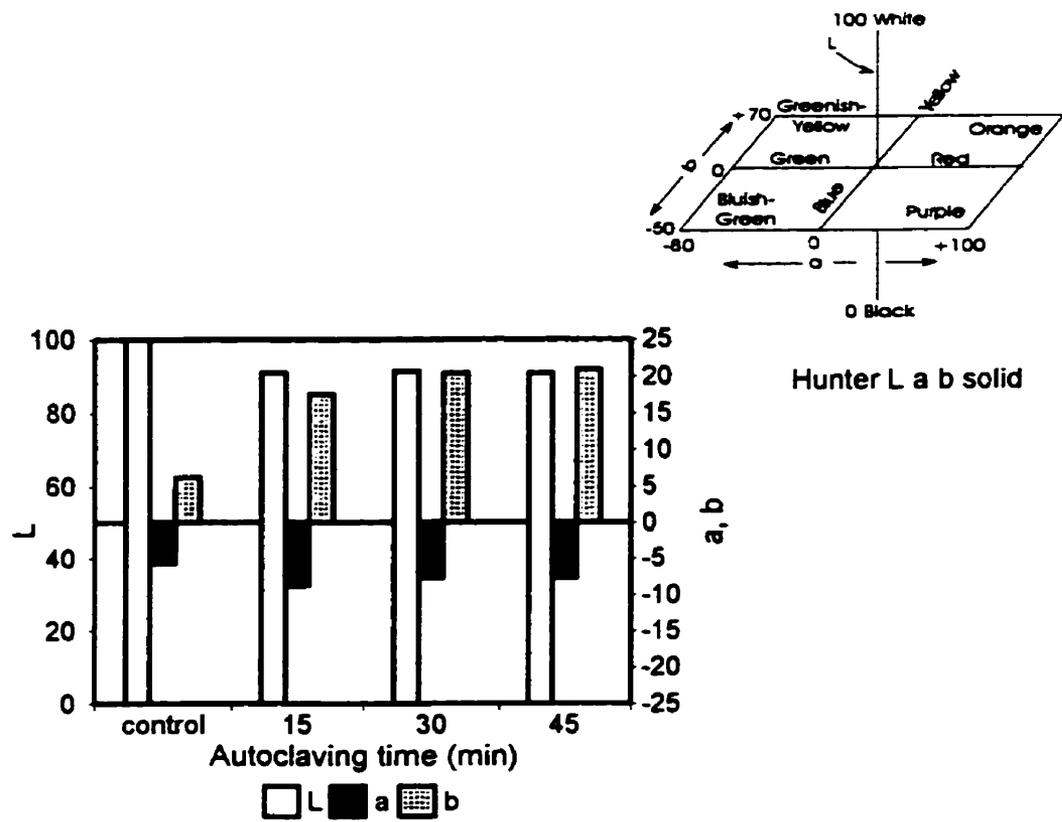
Fig. 2.3. Effect of autoclaving on the Hunter L a b values of a 100 μg/mL sinapic acid solution.

heat treatment time from 15 to 30 min resulted in an increase in the intensity of yellow. However, whiteness and green seemed not to be affected by autoclaving time. In addition, further increases in time after 30 min seemed to have no significant effects on the yellow intensity, b value.

Before the autoclaved solution was analyzed by HPLC, it was filtered through a 0.45 μm filter. A brown solid was retained on the filter. This suggested that some of the colored substances, formed during autoclaving, were water insoluble. Therefore, this filtration was used as a technique to separate the brown solid from the yellow liquid, as indicated in Fig.2.1.

B. Analysis of the brown solid phase

HPLC analysis. HPLC chromatograms of standard sinapic acid and the colored substances in the brown solid phase are shown in Fig.2.4a-b. These substances were dissolved in methanol for the HPLC analysis. Fig. 2.4a shows the HPLC chromatogram of the standard sinapic acid and Fig.2.4b that of the brown solid. A number of peaks were present in the HPLC chromatogram(Fig.2.4b), suggesting the presence of several new compounds. Determination of the structures of these compounds required further experimentation.

Thin layer Chromatography. Based on the HPLC results indicating that there were several compounds present in the solid phase after filtration (Fig.2.4a-b), thin layer chromatography was used to separate three strong bands, which were yellow, orange and purple (Fig.2.5). These three bands had retention factors (Rf) of 0.95±0.01, 0.91±0.01, and 0.72±0.02, respectively, with the yellow band being the major fraction. In addition, it was
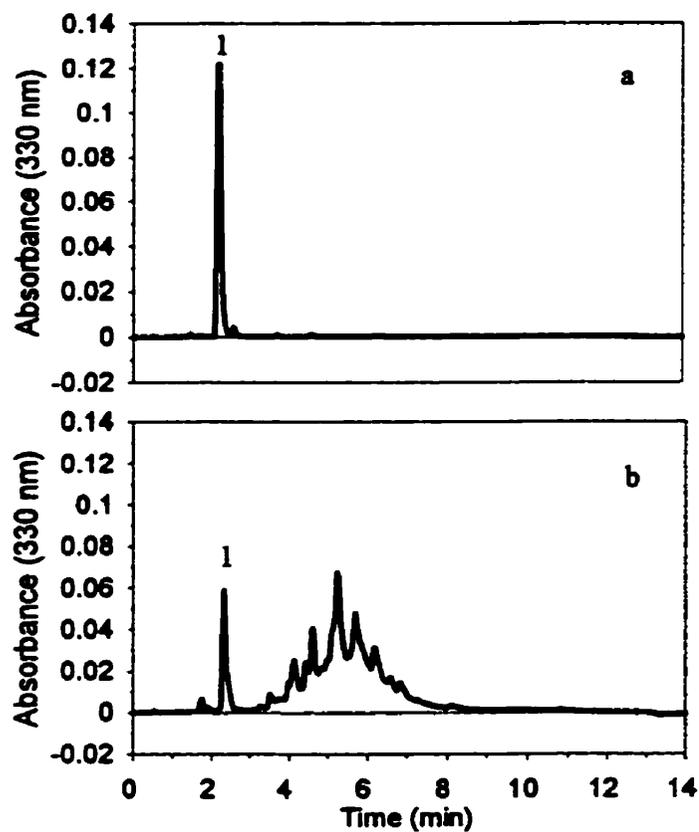
Fig. 2.4a-b. HPLC chromatograms of the solid phase. Samples were applied to HPLC column as methanol solutions. (a) control sinapic acid, (b) brown solid separated by filtering through a 0.45 μm filter. 1. Sinapic acid, other peaks were unidentified.

noted that the yellow and orange compounds were UV light sensitive and turned purple when exposed to UV light for a few minutes. There were also some other faintly colored bands found using TLC analysis. The compounds found in the TLC analysis do not necessarily correspond to the compounds detected by HPLC.
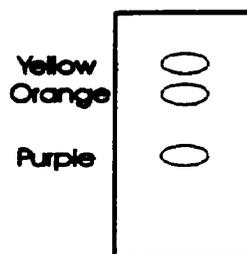
Fig.2.5. Thin layer chromatography of the solid phase
with 100% ethyl acetate as eluant.

NMR analysis. The results of NMR analysis of the yellow, orange, and purple bands gave poorly resolved spectra with many peaks. The only conclusion that could be drawn was that the yellow substance seemed to be a dimer of sinapic acid. There appeared to be two sets of signals for the protons on the aromatic ring and two sets of signals for the methoxyl groups for the yellow substance in comparison with only one set of each of these signals for the sinapic acid. It was also possible that the yellow substance may be composed of two major products as evidenced by signals in both the aromatic and methoxyl region.

C. Analysis of the yellow liquid phase

HPLC analysis of the liquid phase. The results of the HPLC analysis for the control sinapic acid and the liquid phase of the autoclaved samples after filtration are shown in Fig.2.6a-c. Immediately following autoclaving, a small peak corresponding to thomasidioic acid and an unknown peak were found in the liquid phase (Fig.2.6b). Sinapic acid, however, remained as the predominate component after autoclaving (Fig.2.6b). HPLC results indicated that about 40% of the sinapic acid was lost during autoclaving. Therefore, only part of the sinapic acid was converted to new substances during autoclaving, and these new substances may be present only in small amounts since almost no peak other than sinapic acid stood out. After 20 days at 22 °C, with daily loosening the cap of the small bottle for air, the peak of thomasidioic acid was more pronounced whereas the unknown peak seemed to disappear. The peak height of the sinapic acid also decreased (Fig. 2.6c). It was noted that the pH of the solution increased from pH 4.3 to pH 5.3 during this period of storage. The increase in thomasidioic acid content may be due to the conversion of sinapic acid to thomasidioic acid at higher pH values, although the final pH value was still in the acid region. Rubino et al. (1995) reported the formation of thomasidioic acid when sinapic acid was exposed to aerated neutral or alkaline conditions. In detailed studies on the conversion of sinapic acid to thomasidioic acid, it was shown that oxygen was necessary for the conversion (Rubino et al., 1995; Lee, 1997). During the storage test, air was introduced occasionally. The formation of thomasidioic acid was confirmed by NMR spectroscopy. Some thomasidioic acid was formed during the autoclave treatment, although the mechanism of this conversion is unknown.
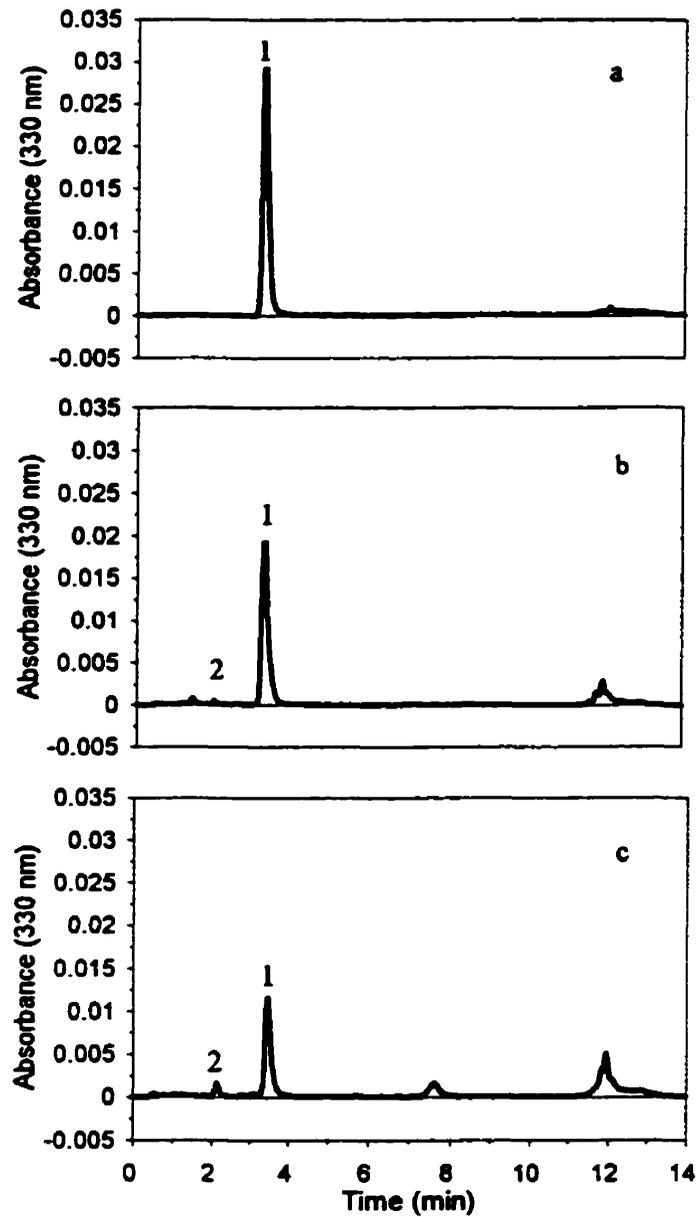
Fig. 2.6a-c. HPLC chromatograms of the yellow liquid phase. Samples were applied to the HPLC column as an aqueous solution. (a) control sinapic acid (sample pH 4.3) and (b) liquid phase separated by filtering through a 0.45 μm filter (sample pH 4.3), (c) sample of (b) after standing at 22 °C for 20 days (sample pH 5.3). 1. Sinapic acid, 2.thomasidioic acid, other peaks were unidentified.

When the sinapic derivatives in the liquid phase were recovered and chromatographed on silica gel, a new substance, syringaldehyde, was identified by ^1H and ^{13}C NMR, by mass spectral analysis and by HPLC analysis of one of the major fractions.

NMR and mass spectra. The identification of syringaldehyde in the liquid phase was made by NMR and mass spectroscopic investigation of the major fraction recovered from silica gel chromatography. NMR spectral data for standard sinapic acid, NMR and mass spectral data for standard syringaldehyde, and the spectral data for the unknown compound present in the liquid are shown in Table 2.1. The NMR spectrum and mass spectral data of the unknown were identical to those of the standard syringaldehyde. This indicated that syringaldehyde had formed during autoclaving.

The identification of syringaldehyde was not readily apparent, since the unknown sample was a mixture of syringaldehyde and several other substances. In fact, the results of ^1H NMR spectrum of the whole residue showed the sample to be the mixture of three major substances, sinapic acid, syringaldehyde and thomasidioic acid. After the residue was recrystallized from CH_2Cl_2 and chromatographed through the silica gel, the major fraction still contained two substances, sinapic acid and syringaldehyde (NMR). This meant that the separation of syringaldehyde from sinapic acid was not complete, although the separation involved several steps. This may be, in part, due to the similarity of the polar nature for both substances. Because of the incomplete separation, the presence of syringaldehyde was not easily confirmed by ^1H NMR spectroscopy alone.

Table 2.1. Nuclear magnetic resonance and mass spectra of standards and compounds produced during autoclaving

Description	Spectrum technique	Spectra
Sinapic acid	^{1}H NMR (Acetone D_6)	7.58 (d, 1H, J=15.9Hz), 7.01 (s, 2H), 6.39 (d, 1H, J=15.8Hz), 3.91 (s, 6H, OCH$_3$)
	^{13}C NMR (Acetone D_6)	167.98 (CO), 148.39 (2C),146.00 (C), 139.20 (C), 126.01 (CH),116.08 (CH), 106.63 (2CH), 56.52 (2CH$_3$)
Syringaldehyde	^{1}H NMR (Acetone D_6)	9.79 (s, 1H), 7.21 (s, 2H), 3.90 (s, 6H, OCH$_3$)
	^{13}C NMR (Acetone D_6)	191.12(CO), 148.94 (2C),142.93 (C), 128.96 (C),107.73 (2CH), 56.63 (2CH$_3$)
	Mass spectrum m/e (relative intensity)	182 (100), 167 (20), 153 (8), 139 (13), 111 (16), 96 (11), 79 (12), 65 (15)
Compound identified	^{1}H NMR (Acetone D_6)	9.81 (s, 1H), 7.23 (s, 2H), 3.92 (s, 6H, OCH$_3$)
	^{13}C NMR (Acetone D_6)	190.93 (CO), 148.84 (2C), 142.82 (C), 128.87 (C), 107.63 (2CH), 56.73 (2CH$_3$)
	Mass spectrum m/e (relative intensity)	182 (100), 167 (20), 153 (7), 139 (13), 111 (20), 96 (9), 79 (15), 65 (18)

However, ^{13}C NMR spectroscopy provided the typical nine-carbon spectrum. In addition, the ^{1}H NMR spectrum exhibited a typical low field aldehyde proton signal. With this information at hand, NMR spectra were examined more closely. Two groups of peaks, representing sinapic acid and syringaldehyde with a mole ratio of around1:0.27, were found in both ^{1}H and ^{13}C NMR spectra for the major fraction from silica gel chromatography. Three

groups of peaks, representing sinapic acid, syringaldehyde and thomasidioic acid with a mole ratio of around 1:0.1:0.03, were found in ^{1}H NMR spectrum of the ethyl acetate extract from the liquid phase. The major fraction from silica gel chromatography was also analyzed using mass spectroscopy. The major mass peaks corresponded to syringaldehyde alone since syringaldehyde has a lower boiling point than does sinapic acid. Only syringaldehyde was evaporated and recorded in the spectrum under the test condition. The spectrum of the unknown was almost perfectly matched with that of the standard. The unknown was, therefore, identified to be syringaldehyde.

HPLC analysis of the major fraction from silica gel chromatography. Based on NMR and mass spectral results, HPLC analysis was conducted to confirm the presence of syringaldehyde in the liquid phase. To ensure that the formation of syringaldehyde was not due to other treatments following the autoclaving, a blank control sinapic acid was also analyzed. Fig.2.7a-d shows the results of HPLC analysis for a recrystallized control sinapic acid (a), a standard sample of syringaldehyde (b), the mixture of the standard sinapic acid and the standard syringaldehyde (c), and the unknown sample from the column chromatographic step (d). Two peaks were found in the unknown sample, which were identified as sinapic acid and syringaldehyde. The identification was done by comparing the chromatogram of the unknown sample (Fig.2.7d) with that of the mixture of the standard sinapic acid and the standard syringaldehyde (Fig.2.7c). A sinapic acid standard (not shown, result similar to Fig.2.7a) and a syringaldehyde standard (Fig.2.7b) were also examined separately to ensure that no interaction of the two substances was interfering with the elution times. To prove that

Fig. 2.7a-d. HPLC chromatograms of the major fraction from silica gel
chromatography (a) sinapic acid blank, (b) standard syringaldehyde, (c)
mixture of standard sinapic acid and syringaldehyde and, (d) unknown
sample identified to be a mixture of sinapic acid and syringaldehyde. 1.
Sinapic acid, 2. Syringaldehyde.

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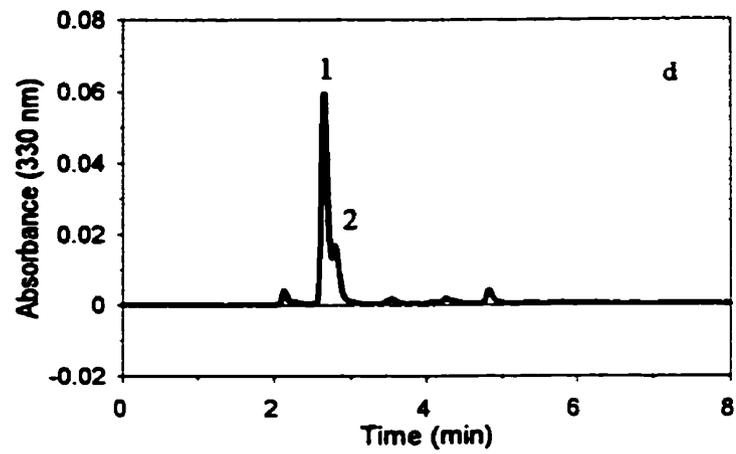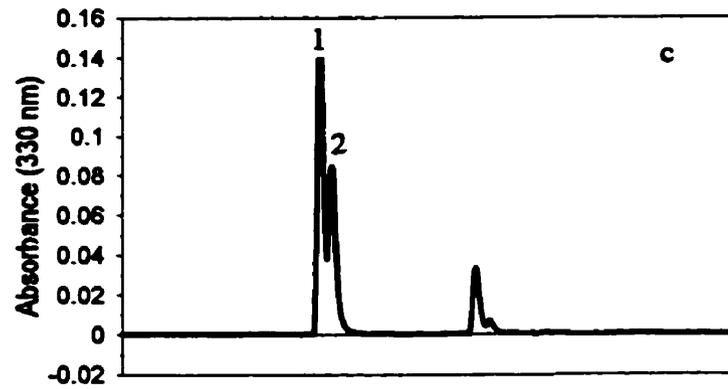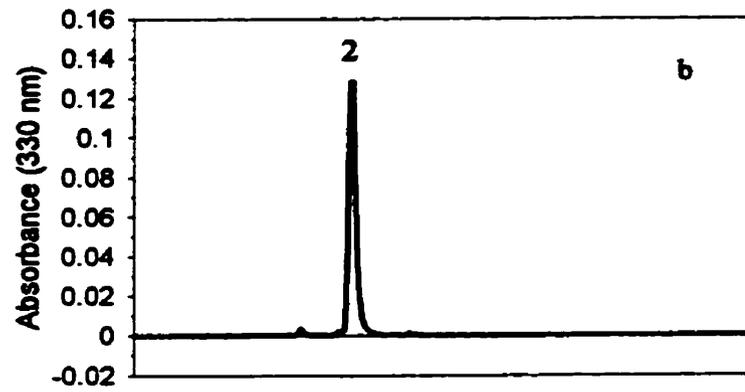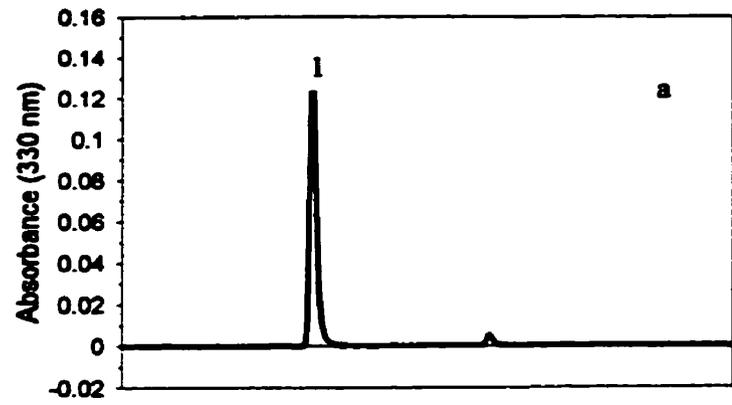
syringaldehyde was produced during autoclaving but not during the sample preparation following the autocalving, the standard sinapic acid was used as a blank sample and run through the same experimental procedure but without autoclaving. To do this, the sinapic acid was dissolved in water, then recovered by ethyl acetate extraction followed by recrystallization from CH_2Cl_2. After removing the solvent under vacuum, the substance was analyzed by HPLC. The HPLC chromatogram of this blank sinapic acid is given in Fig.2.7a. The single peak was identified as sinapic acid. There was no evidence of syringaldehyde in the control sample. This proved that syringaldehyde was formed during autoclaving. Attempts were made to improve resolution of the HPLC analysis by using two other gradients for the mixture of sinapic acid and syringaldehyde. However, similar results with no improvement in resolution were obtained. Further experimentations for this purpose were terminated since the presence of syringaldehyde was firmly confirmed based on NMR and MS analysis. HPLC analysis was mainly conducted to confirm the results of the NMR and MS analysis. Thomasidioic acid found in the previous HPLC chromatograms (Fig. 2.6.a-c) was not detected in the major fraction from silica gel column chromatography. This may indicate a good separation between thomasidioic acid and syringaldehyde during the silica gel chromatography.

Retention time for syringaldehyde was very close to that for sinapic acid. In addition, the concentration of syringaldehyde in the liquid phase was low. It was estimated from HPLC data that 40% of the sinapic acid was converted to other substances during a 30-min autoclaving. The starting material was a 100 µg/mL sinapic acid aqueous solution. The

autoclaved solution would contain about 60 µg/mL sinapic acid, about 6 µg/mL syringaldehyde, about 2 µg/mL thomasidioic acid (HPLC, NMR), and the rest were the undetermined substances. The earlier HPLC analysis of the yellow liquid phase (Fig. 2.6b-c) did not show the presence of syringaldehyde. This is due to the excess of sinapic acid present, that masks the signal from syringaldehyde due to their similar retention time.

Color significance of syringaldehyde. In order to evaluate the color significance of syringaldehyde, the UV and transmittance spectra of syringaldehyde were compared with those of the sinapic acid (Fig.2.8a-b). The UV spectra for both substances are shown in Fig.2.8a. Syringaldehyde had a spectrum with two maxima around 220 and 310 nm. Both maxima are at a lower wavelength than those of the sinapic acid. While sinapic acid shows almost no absorbance in the wavelength region from 375 to 400 nm, syringaldehyde shows a slight absorbance in this region. This is consistent with the dull yellow appearance of syringaldehyde. The transmittance spectra for syringaldehyde and sinapic acid are shown in Fig.2.8b. Syringaldehyde shows a relatively lower percentage transmittance than does sinapic acid in the whole visual light region, especially in the wavelength region from 380 to 400 nm. Therefore, the formation of syringaldehyde would contribute a yellowness and a darkness to the sinapic acid solution. However, comparison of the transmittance spectrum of syringaldehyde solution (Fig.2.8b) with that of the autoclaved sinapic acid solution (Fig.2.2b), leads to the conclusion that syringaldehyde contributes only a very small amount of yellowness and darkness to the solution. Other undetermined components, especially those three colored substances shown by the thin layer chromatography, could be the major
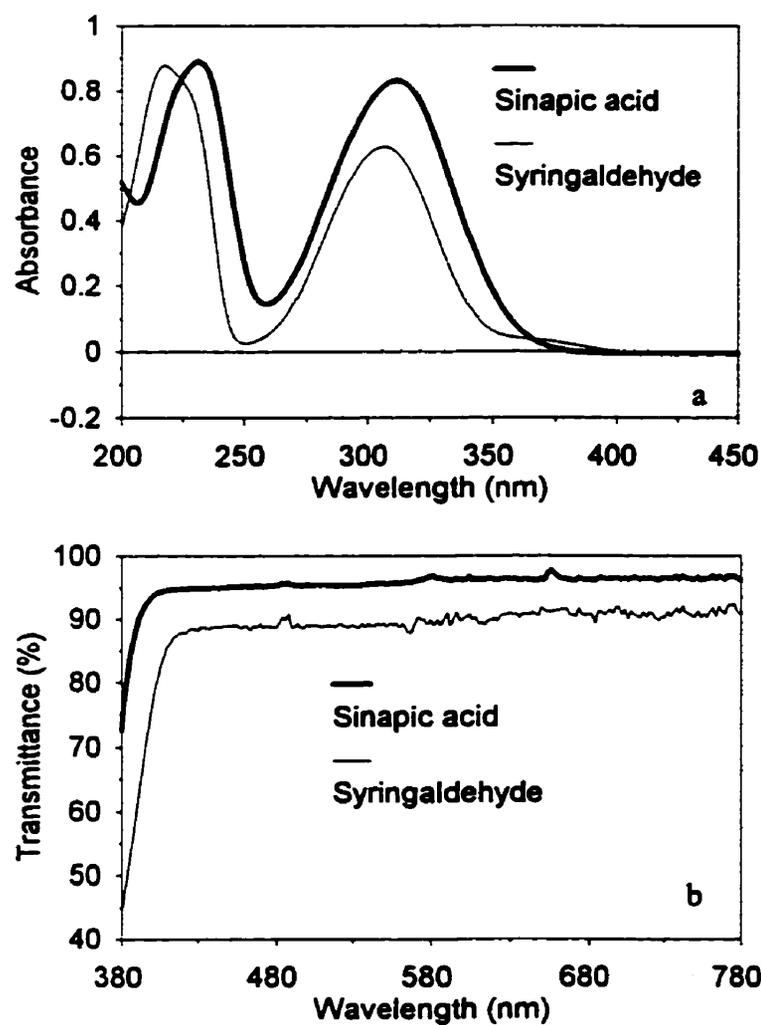
Fig.2.8a-b. Comparison of UV spectra (dilute 10 times, a) and visual transmittance spectra (b) of a 100 μg/mL syringaldehyde and a 100 μg/mL sinapic acid aqueous solutions.

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components responsible for color change of sinapic acid during autoclaving. The concentration of these highly colored compounds may be much lower than that of syringaldehyde.

Sinapine

A. Spectral analysis

The results of UV and visual spectral analysis for sinapine solution before and after autoclaving are shown in Fig.2.9a-b. Spectra of sinapine for the control and autoclaved sample were perfectly matched. This would suggest that no changes occurred during autoclaving.

B. HPLC analysis

Both control and autoclaved sinapine solutions (100 μg/mL) were analyzed using HPLC for a storage period of 20 days at 22 °C with a daily loosening of the cap of the small bottle for air. No differences were found between the two groups of samples (Appendix 2.5). This also suggested that no structural changes occurred during autoclaving.

In conclusion, it has been demonstrated that a yellow coloration developed when a 100 μg/mL sinapic aqueous solution was autoclaved for 15 min at 121 °C and 0.1 MPa. A newly formed yellow substance was identified to be syringaldehyde by NMR, mass spectrum and HPLC analysis. Thomasidioic acid was also formed during autoclaving. There were at least three other colored substances estimated to be present by TLC analysis. However, their
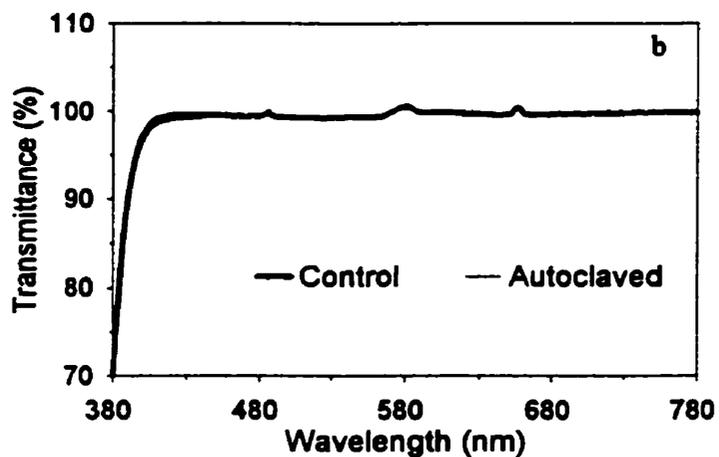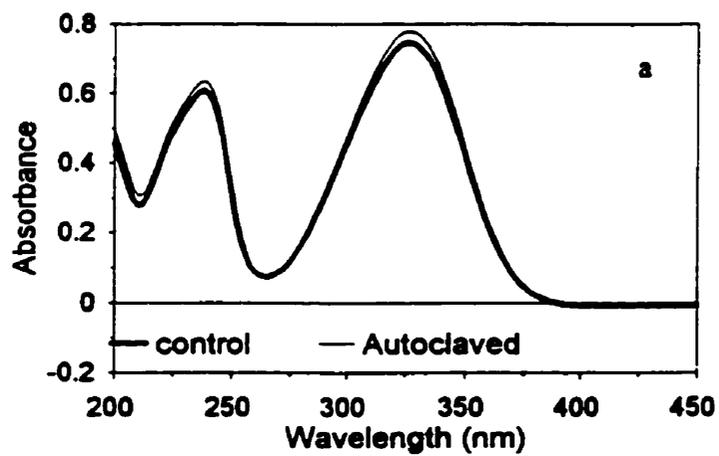
Fig. 2.9a-b. Effect of autoclaving on the UV spectrum (diluted 10 times, a) and visual transmittance spectrum (b) of a 100 μg/mL sinapine aqueous solution.

identities have not been determined. The structural and color changes of a 100 μg/mL sinapic

acid aqueous solution during autoclaving are summarized in Fig.2.10. Sinapine, under the

same conditions, showed no evidences of any structural or color changes.
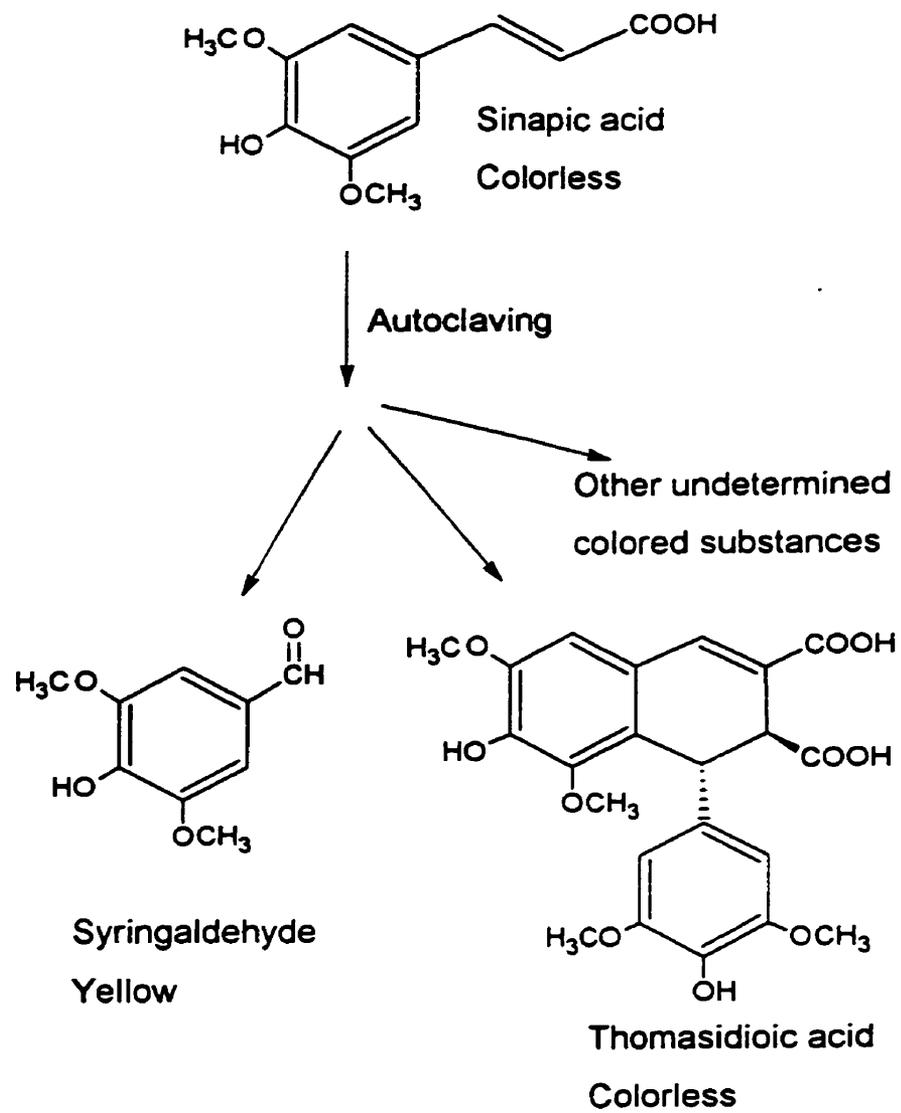
Fig. 2.10. Structural and color changes of sinapic acid during autoclaving.

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CHAPTER III. STRUCTURAL CHANGES OF SINAPIC ACID DURING ALKALI INDUCED AIR OXIDATION WITH RESPECT TO THE DEVELOPMENT OF COLORED SUBSTANCES

ABSTRACT

Structural changes of sinapic acid were induced by air oxidation in aqueous solutions at pH 7 - 10 and followed by spectral and high performance liquid chromatographic (HPLC) analysis. Color properties of the sinapic acid solutions were determined by taking the transmittance spectra, calculating the Commission Internationale de l' Eclairage (CIE) 1931 tristimulus values and converting to Hunter L a b values. Reaction rate constants for sinapic acid were determined by a kinetic study based on the quantitative results from HPLC analysis. These reactions were first order with respect to sinapic acid and fit the appropriate equation with a coefficient of $R^2 > 0.97$. Sinapic acid was converted to thomasidioic acid with reaction rate constants (k) of 8.54×10^{-6}, 2.51×10^{-5} and 4.87×10^{-5} s^{-1} in phosphate-boric acid buffers of pH 7, 8.5 and 10, respectively. Similar reactions in ammonium bicarbonate buffers were more than ten times faster. With time, thomasidioic acid further converted to 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid. Air oxidation of sinapic acid aqueous solutions caused darkening of the color for the system, with the 2,6-dimethoxy-p-benzoquinone being one major color contributor.

KEY WORDS: Canola phenolics, chromatographic analysis, color, 2,6-dimethoxy-p-benzoquinone, 6-hydroxy-5,7-dimethoxy-2-naphthoic acid, reaction rate constant, sinapic acid, thomasidioic acid

INTRODUCTION

With an annual global production of more than 27 million metric tons (Eskin et al., 1996), canola/rapeseed represents one of the most important oilseeds in the world (Shahidi, 1990). There is an interest in preparing a food grade protein from canola meal (Ismond and Welsh, 1992; Gillberg and Tornell, 1976a; Owen et al., 1971), especially when it was found that canola meal was well balanced in its amino acid composition (Liu et al., 1982; Ohlson and Anjou, 1976). However, the utilization of canola/rapeseed protein in human foods has been limited by the presence of antinutritional factors such as glucosinolates, phytates and phenolics (Shahidi and Naczk, 1995). Among them, phenolics have been the subject of many studies due to their contributions to the dark color, bitter taste and the astringency of rapeseed/canola meal (Shahidi and Naczk, 1992) as well as their detrimental effect on the gelation property of canola protein (Rubino et al., 1996b). Canola phenolics are present in free, esterified, and bound forms (Krygier et al, 1982a). Canola flour contains from 91 to 93.5% of their phenolic acid in the esterified form (Shahidi and Naczk, 1992). Sinapine, the choline ester of sinapic acid, is the major esterified phenolic acid (Blair and Reichert, 1984; Fenton et al, 1980; Austin and Wolff, 1968), whereas sinapic acid represents a high proportion of the free phenolic acid and 99% of the phenolic acids released from hydrolysis of the esters in the flour (Krygier et al., 1982b).

The dark color of the canola meal is one of the major problems that limits the

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utilization of canola protein (Youngs, 1991). A dark colored meal is produced after oil

extraction (Youngs, 1991). During protein isolation, conditions of high pH produce a darker

protein isolate than do conditions of low pH (Keshavarz et al., 1977). Although the

observations of a dark meal or protein isolate have been frequently reported (Youngs, 1991;

Keshavarz et al., 1977) and more or less associated with phenolics (Shahidi and Naczk, 1995;

1992), detailed information underlying the reactions responsible for the development of

colored substances is not available. Such information, however, should indicate the

mechanisms by which the colored substances develop and, therefore, is important to the

oilseed processors.

Although no relationship has ever been made between the structural changes of the

phenolics and color properties of canola/rapeseed protein, the structural changes of sinapic

acid in air saturated basic conditions have been observed by several researchers (Charlton and

Lee, 1997; Rubino et al., 1995; 1996a; Bouchereau et al., 1992). Rubino et al. (1995)

reported the formation of thomasidioic acid under these conditions showing that the

conversion was oxygen dependent (Rubino et al., 1996a). Charlton and Lee (1997) reported

the formation of 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic

acid from sinapic acid via thomasidioic acid at pH 13 in the presence of oxygen. With HPLC

analysis, Bouchereau et al. (1992) detected new compounds derived from rapeseed phenolics

in the methanol extract of rapeseed flour, although the compounds were not identified. In a

study looking at the effect of processing conditions on phenolic content, treatments,

especially heating, have been shown to decrease the sinapine content and increase the lignan

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amount (Jensen, 1990). Although the oxidation of sinapic acid under alkaline conditions is known to lead to thomasidioic acid, 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid, the color properties of these oxidation products have not been determined. Reaction rate constants for these conversions under different pH conditions have also not been determined, which should provide useful information when considering protein isolation under alkaline conditions.

As part of a series of investigations looking at the contributions of phenolics to the color property of canola protein, structural changes of sinapic acid were induced by air oxidation at pH 7-10 and followed by spectral and HPLC analysis for a period of 10 days. Reaction rate constants were determined at pHs of 7, 8.5 and 10. Color properties of solutions were also determined in order to evaluate the color contributions of the new substances to the system.

CHAPTER III. ALKALI EFFECT FOR SINAPIC ACID

MATERIALS AND METHODS

A. Sources of Materials

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetic acid and sodium hydroxide used for HPLC were verified ACS-grade and purchased from Fisher Scientific Co. (Nepean, ON, Canada). Other chemicals used for HPLC were HPLC-grade. All other chemicals, unless stated otherwise, were verified ACS-grade and purchased from Fisher Scientific Co.

Thomasidioic acid was prepared according to the procedure outlined by Lee (1997) and Rubino et al. (1995), which involved the oxidation of sinapic acid in an ammonium bicarbonate buffer (pH 8.5). The crude product was recrystalized from acetone several times to yield thomasidioic acid as a colorless solid (Ahmed et al., 1973). This purified thomasidioic acid was used as a standard for identifying thomasidioic acid formed from sinapic acid. 2,6-Dimethoxy-p-benzoquinone was prepared according to the method outlined by Lee (1997). Chromium trioxide (634 mg, 6.34 mmol) was dissolved in water (1 mL) and acetic acid (18 mL). 2,6-Dimethoxyphenol (206 mg, 1.34 mmol) was added and the solution stirred at room temperature for 90 minutes. Water (30 mL) was added and then the solution extracted with CH_2Cl_2 (3x10 mL). The organic layers (lower layers) were combined and washed with sodium bisulfite solution (5%, 2x20 mL) and water (2x20 mL). The solution was dried with $MgSO_4$ and evaporated under vacuum to yield a bright yellow solid. The solid was

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filtered through silica gel with ethyl acetate to give the 2,6-dimethoxy-*p*-benzoquinone as a yellow solid. 6-Hydroxy-5,7-dimethoxy-2-naphthoic acid was also prepared using the method of Lee (1997) by air oxidation of sinapic acid in strongly basic solution (pH 13). 2,6-Dimethoxy-p-benzoquinone was reported as a yellow compound, while 6-hydroxy-5,7-dimethoxy-2-naphthoic acid was reported as pale tan needles from MeOH-H_2O (Hostettler and Seikel, 1969).

B. Sample Preparation

Phosphate-boric acid buffers were prepared according to Britton and Robinson (1931). The stock buffer solution was composed of an equal mixture of dihydro monosodium phosphate, monohydro disodium phosphate and boric acid, each 0.04 M. Three buffers of pH 7, 8.5 and 10 were prepared from the stock solution by adding 0.2 N sodium hydroxide solution. Ammonium bicarbonate buffers (0.12 M) were prepared according to Lee (1997) and adjusted to pH 8.5 and 10 using ammonium hydroxide. Experiments in this buffer system were conducted to complement the phosphate-boric acid system. Results in phosphate-boric acid buffers constituted the main body of work in this paper.

A sinapic acid solution (200 µg/mL) was prepared with deionized water. Three samples of 4 mL each in phosphate-boric acid buffer of pH 7, 8.5 and 10 (two samples at pH 8.5 and 10 in ammonium bicarbonate buffers), respectively, were prepared by combining 2 mL of the sinapic acid solution with 2 mL of each buffer solution of different pHs so that 100 µg/mL (0.446 mmol) sinapic acid solutions with different pHs were obtained. These solutions

were stirred in air at room temperature (22 °C). Oxygen is needed for the conversion of

sinapic acid to thomasidioic acid (Rubino et al., 1996) and the conversion of thomasidioic acid

to 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid (Charlton

and Lee, 1997). Water vapor loss was determined to be 1% per day under the test condition.

This loss was ignored during the kinetic study. The pH values of these solutions were checked

before each analysis was made. Only the pH value of the phosphate-boric acid solution at pH

10 had a slight deviation (decreased) and was readjusted by adding sodium hydroxide.

Spectral and HPLC analysis were carried out for a period of 10 days. For HPLC, a sample

size of 1 μL was used for the kinetic study whereas 25 μL was injected during the

identification of the p-benzoquinone and the 2-naphthoic acid using a 25 μL sample loop.

Three samples of pH 7, 8.5 and 10 for ultraviolet spectral analysis were prepared by a 20-fold

dilution of the sinapic acid solution (200 μg/mL) with phosphate-boric acid buffers of

different pHs and stirred in air at room temperature (22 °C). For the spectral and HPLC

analyses of the standard thomasidioic acid, 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-

5,7-dimethoxy-2-naphthoic acid, these compounds were dissolved directly in the appropriate

buffers just before the measurement.

C. HPLC Analysis

Chromatographic equipment consisted of two Waters (Milford, MA) pumps (model

501 and 510) and an automated gradient controller model 680, a Shimadzu (Kyoto, Japan)

SPD-6A ultraviolet (UV) spectrophotometric detector, and a Hewlett-Packard (Avondale,

PA) model HP3396II integrator. A reverse-phase C18 column (Supelcosyl, 3-µm particle size, 33x4.6 mm i.d.; Supelco, Bellefonte, PA) was used. Component A was a 0.05 M acetate buffer prepared by a 1:100 dilution of a stock pH 4.7 acetate buffer. The stock buffer was prepared by adjusting 5 M acetic acid to pH 4.7 with solid sodium hydroxide (Hagerman and Nicholson, 1982). Component A was filtered through a 0.45 µm filter. Component B was 100% HPLC-grade methanol. The column was maintained at 37 °C and run at a constant flow rate of 1.4 mL/min.

The initial elution solvent was 15% methanol and 85% component A. After a 12-min isocratic flow, a 2-min linear gradient was used to change the solvent composition to 100% methanol. This composition was maintained for 2 min, after which another 2-min linear gradient returned the solvent to its original composition.

D. Kinetic Study

Reaction rate constants (k) were obtained by a kinetic study based on the time dependent change in concentration of sinapic acid as determined from the peak areas of the HPLC chromatograms.

For each reaction at pH 7, 8.5 and 10, the concentration of sinapic acid was determined about ten times by HPLC during the course of the reaction (0 to 35 hours). The HPLC response (peak area) to sinapic acid was calibrated using standardized solutions of sinapic acid (2.5 to 200 µg/mL). Each of the three groups of reactant time dependent concentrations at different pHs was fit to a first order reaction equation:

$$\ln\frac{c_o}{c} = kt$$

and a second order equation:

$$\frac{1}{c} - \frac{1}{c_o} = kt$$

where c_o and c were the initial concentration (mol/L) and the concentration at time t, t is the reaction time (s), and k is the reaction rate constant, (s^{-1}) in the first order equation, and $(Lmol^{-1}s^{-1})$ in the second order equation.

If the reaction is of first order, the plot of $\ln(1/c)$ versus t should give a straight line. Similarly, if the reaction is of second order, the plot of $1/c$ versus t should give a straight line. A linear regression was conducted for each group of sinapic acid concentrations. R^2 (R=correlation coefficient) was used to judge the linear relationship between the independent value t and the dependent values $\ln(1/c)$ or $1/c$ and, therefore, to determine which equation best fit the data (Barrow, 1979). The equation best able to fit the data was used to calculate the reaction rate constant (k) and the theoretical concentrations of reactant for the kinetic curves.

E. Spectral analysis and color determination

Spectral analysis and color determination followed the same procedure as in previous reports (Chapter II, Cai et al., 1999a).

RESULTS AND DISCUSSION

A. Structural Changes

1. HPLC Analysis

The HPLC chromatograms shown in Fig.3.1a-c indicate that virtually all the sinapic acid was converted to thomasidioic acid during the alkali induced air oxidation at pH 7, 8.5 or 10 (detector wavelength 330 nm). With the reaction at pH 7 (Fig.3.1a), there was 50% conversion to thomasidioic acid after 24 hours. The conversion was not complete until after about 169 hours. With the reaction at pH 8.5 (Fig.3.1b), thomasidioic acid was detected after two hours and there was 91% conversion to thomasidioic acid after 24 hours. With the reaction at pH 10 (Fig.3.1c), there was about 36% and 90% conversion to thomasidioic acid after two and ten hours, respectively. The conversion was nearly complete within 24 hours (Fig.3.1c). It was noted that during the period from 24 hours to 169 hours, the peak of the thomasidioic acid became smaller while a new peak identified as 6-hydroxy-5,7-dimethoxy-2-naphthoic acid appeared. Structural changes of sinapic acid were similar for all three pH conditions but the conversions were slower at lower pH. These HPLC chromatograms as well as additional data points also provided kinetic data for the conversion of sinapic acid to thomasidioic acid at different pHs.

The air oxidative conversion of sinapic acid to 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid via thomasidioic acid at high pH has been previously
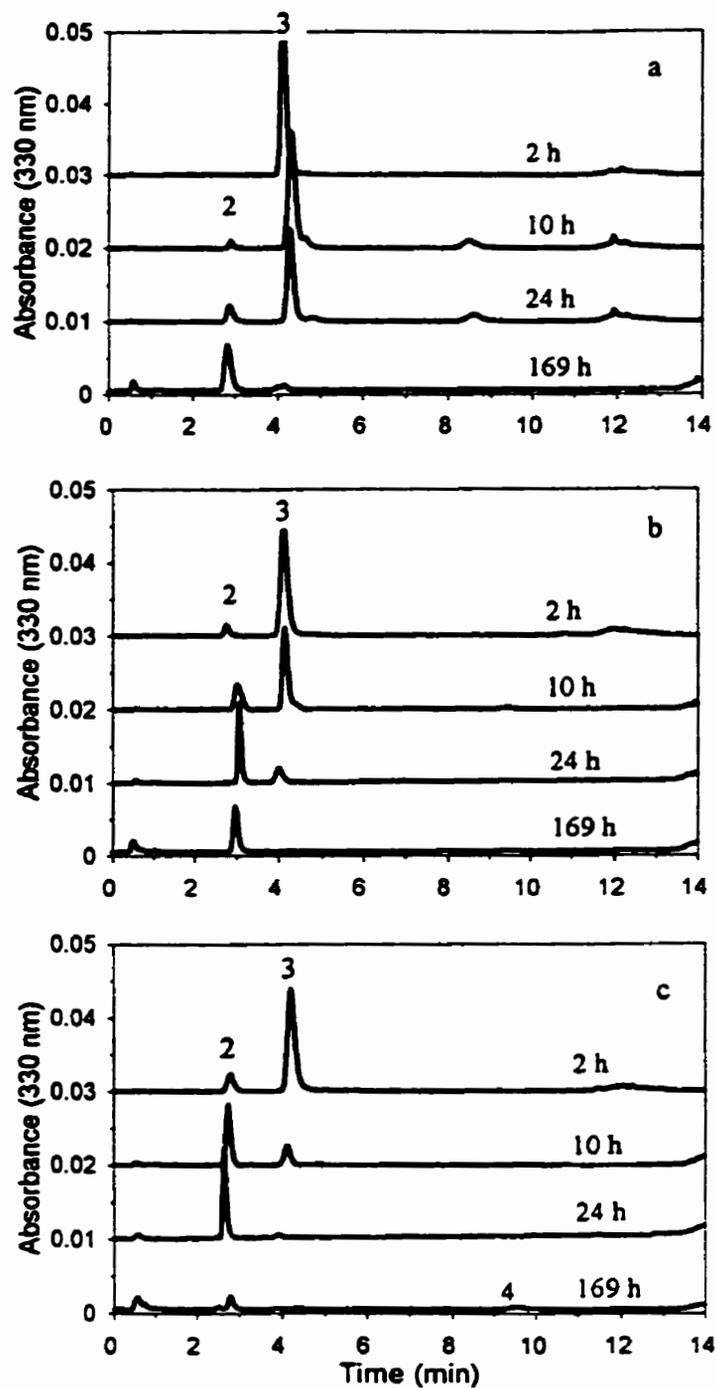
Fig.3.1a-c. HPLC chromatograms of the sinapic acid reaction products with reactions
conducted in phosphate-boric acid buffer of (a) pH7, (b) pH 8.5 and (c) pH 10. 2.
Thomasidioic acid, 3. sinapic acid, 4. 6-hydroxy-5,7-dimethoxy-2-naphthoic acid.

reported (Charlton and Lee, 1997). In the present study, these two oxidation products were determined to be present in samples at all three pHs (7, 8.5 and 10) after 169 hours. The expanded HPLC chromatograms of the three air oxidation samples after 169 hours are shown in Fig.3.2. Peaks 1 and 4 were identified as 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid, respectively. Peak 4 was more pronounced at pH 10 than pH 8.5 and pH 7, whereas peak 1 was similar at all three pH values. This may suggest that 2,6-dimethoxy-p-benzoquinone was unstable and did not accumulate during the reaction. The fact that the 6-hydroxy-5,7-dimethoxy-2-naphthoic acid peak increased while the thomasidioic acid peak decreased (Fig.3.1c) was consistent with the known oxidation of thomasidioic acid to 6-hydroxy-5,7-dimethoxy-2-naphthoic acid (Charlton and Lee, 1997).

Similar reactions were found in ammonium bicarbonate buffers at pH 8.5 and pH 10, but the reaction rates were more than ten times faster.

HPLC analyses of a sample of sinapic acid, air oxidized at pH 8.5 for 240 hours, using detector wavelengths of 330 and 400 nm are given in Fig.3.3a-b. Analysis at 400 nm is more sensitive to colored compounds such as 2,6-dimethoxy-p-benzoquinone. Note that the pH of the HPLC solvent is 4.7 and that the visual and UV spectral properties of the products at this pH will be different than at higher pH. At higher pH (7-10), ionization of the products at the phenolic hydroxyls may produce anions with absorption in the visible region of the spectrum. Results for samples oxidized at pH 7, and pH 10 and analyzed at pH 4.7 were similar. Peaks 1 and 4 were identified to be 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid by comparing the chromatograms of the samples with those of the standards.
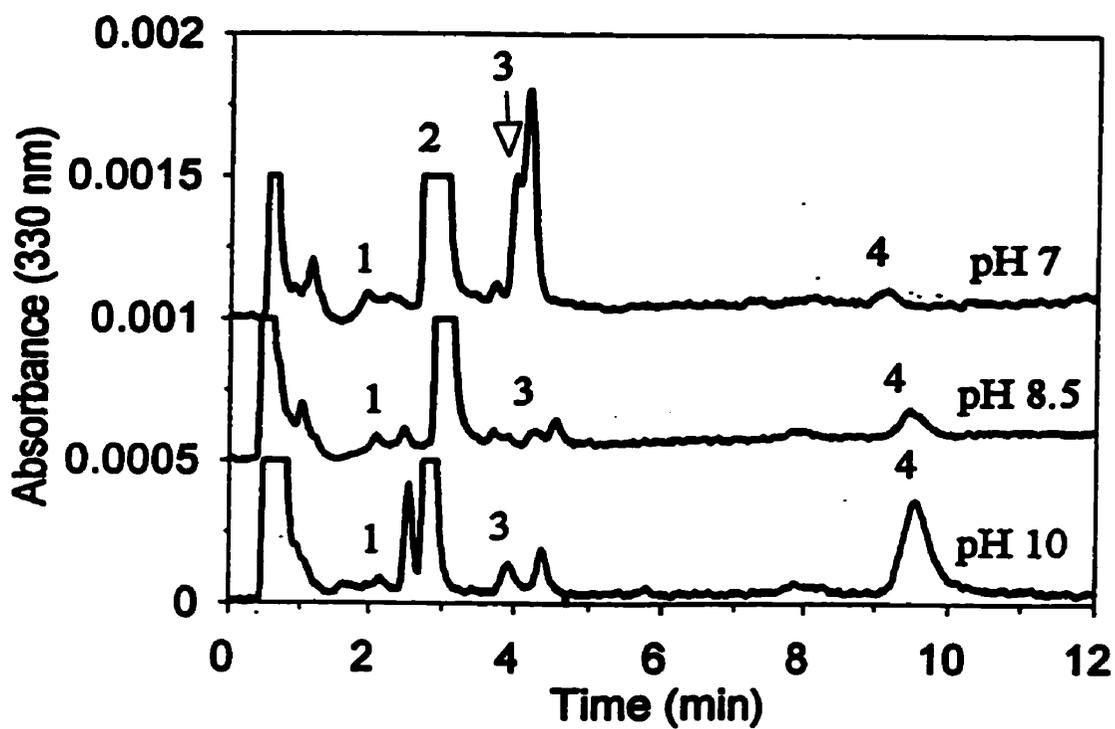
Fig. 3.2. Expended HPLC chromatograms of the sinapic acid reaction products after 169 hours. 1. 2,6-dimethoxy-*p*-benzoquinone, 2. Thomasidioic acid, 3. sinapic acid, 4. 6-hydroxy-5,7-dimethoxy-2-naphthoic acid. Other peaks were unidentified.
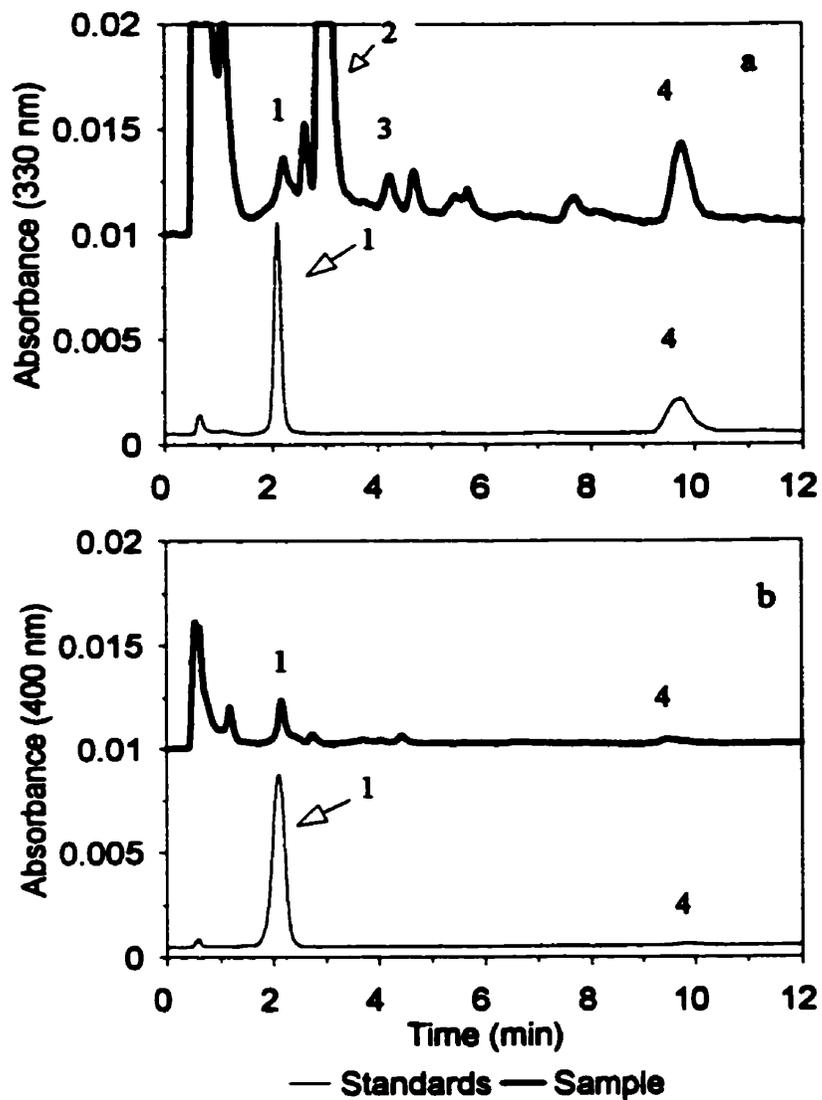
Fig.3.3a-b. HPLC chromatograms of a mixture of standard 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic plus the sinapic acid reaction products in phosphate-boric acid buffer of pH 8.5 after 240 hours at (a) 330 nm and (b) 400 nm. 1. 2,6-dimethoxy-p-benzoquinone, 2. Thomasidioic acid, 3. sinapic acid, 4. 6-hydroxy-5,7-dimethoxy-2-naphthoic acid. Other peaks were unidentified.

This identification was also confirmed by spiking the sample with the standard p-benzo-quinone and the 2-naphthoic acid. The high absorbance at 400 nm for the p-benzoquinone indicated it to be a strongly colored substance. On the other hand, the 2-naphthoic acid had a very low absorbance at 400 nm, indicating it to be a substance of limited color.

The amounts of 2,6-dimethoxy-p-benzoquinone and the 6-hydroxy-5,7-dimethoxy-2-naphthoic acid were estimated to be 0.042 and 0.121 mmol/L, a mole ratio of 1:2.9, after air oxidation for 240 hours at pH 8.5. This ratio is less than the theoretical mole ratio of 1:1, which would result if equal moles of the two compounds were produced as the only reaction products from thomasidioic acid.

2. UV spectrum

The UV spectra of 10 μg/mL sinapic acid solutions after air oxidation for 0 and 24 hours are shown in Fig.3.4a-b. The UV spectra of 10 μg/mL standard thomasidioic acid solutions of different pHs are given in Fig.3.4c. At zero time, increasing pH caused a strong bathochromic shift, the shift of the maximum absorbance toward higher wavelengths, relative to the spectrum recorded at pH 7 (Hendrickson et al., 1970). The effect of alkaline conditions on the absorption properties of phenolic compounds has been reported by several researchers (Ribereau-Gayon, 1972; Harborne, 1964). Large bathochromic shifts were noted in most cases under basic conditions (Harborne, 1964). The pKa values of the carboxylic group and hydroxy group of sinapic acid are 4.47 and 9.21, respectively (Smyk and Drabent, 1989). Therefore, at pH 7 only the carboxylic group is ionized (anion I). At pH 10, both the
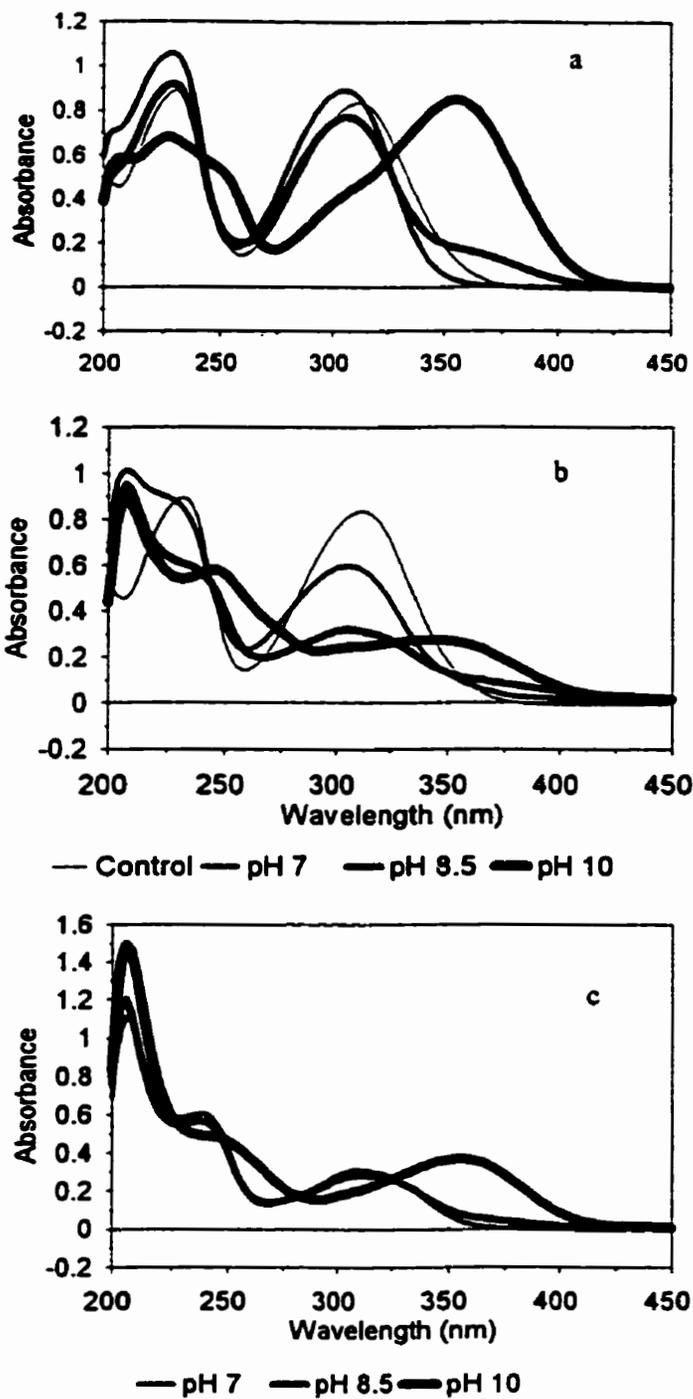
Fig.3.4a-c. UV spectra of a 10 μg/mL phosphate-boric acid buffer solution of pH 7, 8.5 and 10. (a) sinapic acid after 0 hour, (b) sinapic acid after 24 hours and (c) standard thomasidioic acid. Control was sinapic acid in deionized water with a natural pH of 4.3.

carboxylic and the hydroxy groups are ionized (anion II) (Fig.3.11). The strongest

bathochromic shifts occur on ionization of the phenolic hydroxyl. After 24 hours (Fig.3.4b),

results of the UV spectrum showed that the absorbances of sinapic acid of different anionic

forms (anion I 305 nm and anion II 355 nm) had decreased as expected. The UV absorbance

of the solutions at pH 8.5 and 10 in fact were similar to the spectra of the oxidized product,

thomasidioic acid (Fig.3.4b-c). Unlike the situations at pH 8.5 and 10, the spectrum obtained

after air oxidation at pH 7 for 24 hours did not resemble that of the standard thomasidioic

acid, since only 50% sinapic acid was converted to thomasidioic acid.

B. Kinetic Study of the disappearance of sinapic acid

It was found that the reaction rate data fit a first order equation better than a second

order equation. The correlation coefficients (R^2) for the first order equations were 0.98, 0.99

and 0.99, respectively, for the reactions in phosphate-boric acid buffers of pH 7, 8.5 and 10,

whereas R^2 for the second order equations were 0.65, 0.93 and 0.85, respectively. The first

order equations were, therefore, used to calculate the reaction rate constant (k) values and

the theoretical concentrations of sinapic acid during the reactions.

The reaction equations, reaction constants (k), half lives ($t_{1/2}$)and coefficients (R^2) for

both the phosphate-boric acid buffer and the ammonium bicarbonate buffer are summarized

in Table 3.1. In phosphate-boric acid buffers, the reaction rate constants are 8.54×10^{-6},

2.51×10^{-5} and 4.87×10^{-5} s^{-1} for reactions at pH 7, 8.5 and 10, respectively. The reaction at pH

10 was about twice as fast as the reaction at pH 8.5 and about five times as fast as the

reaction at pH 7. These differences can also be seen from the half lives $(t_{1/2})$ of the reactions, which were 22.50, 7.66 and 3.95 hours for pH 7, 8.5 and 10, respectively. The reactions in ammonium bicarbonate buffers were more than ten times faster with reaction rate constants (k) of 4.30×10^{-4} and 6.18×10^{-4} s^{-1} at pH 8.5 and 10, respectively.

Table 3.1. First order reaction equations, reaction rate constants (k), half lives $(t_{1/2})$ and correlation coefficients R^2 for the reactions of sinapic acid aqueous solutions at pH 7, 8.5 and 10.

pH	Equation	k (s^{-1})	$t_{1/2}$ (h)^a	R^2
	Phosphate-boric acid buffer			
pH 7	$\ln(c_o/c) = 0.038 + 8.54 \times 10^{-6}$ t	8.54×10^{-6}	22.50	0.98
pH 8.5	$\ln(c_o/c) = 0.075 + 2.51 \times 10^{-5}$ t	2.51×10^{-5}	7.66	0.99
PH 10	$\ln(c_o/c) = 0.083 + 4.87 \times 10^{-5}$ t	4.87×10^{-5}	3.95	0.99
	Ammonium bicarbonate buffer			
pH 8.5	$\ln(c_o/c) = 0.088 + 4.30 \times 10^{-4}$ t	4.30×10^{-4}	0.45	0.99
pH 10	$\ln(c_o/c) = -0.177 + 6.18 \times 10^{-4}$ t	6.18×10^{-4}	0.31	0.97

^{a} The half life $(t_{1/2})$ for the reactions was found as:

$t_{1/2} = 0.693/k$

Where $t_{1/2}$ is the half life, k the reaction rate constant.

Linear and logarithmic plots of sinapic acid concentration and the corresponding plots generated from the best fit rate constants are given in Fig.3.5a-b. In general, the theoretical
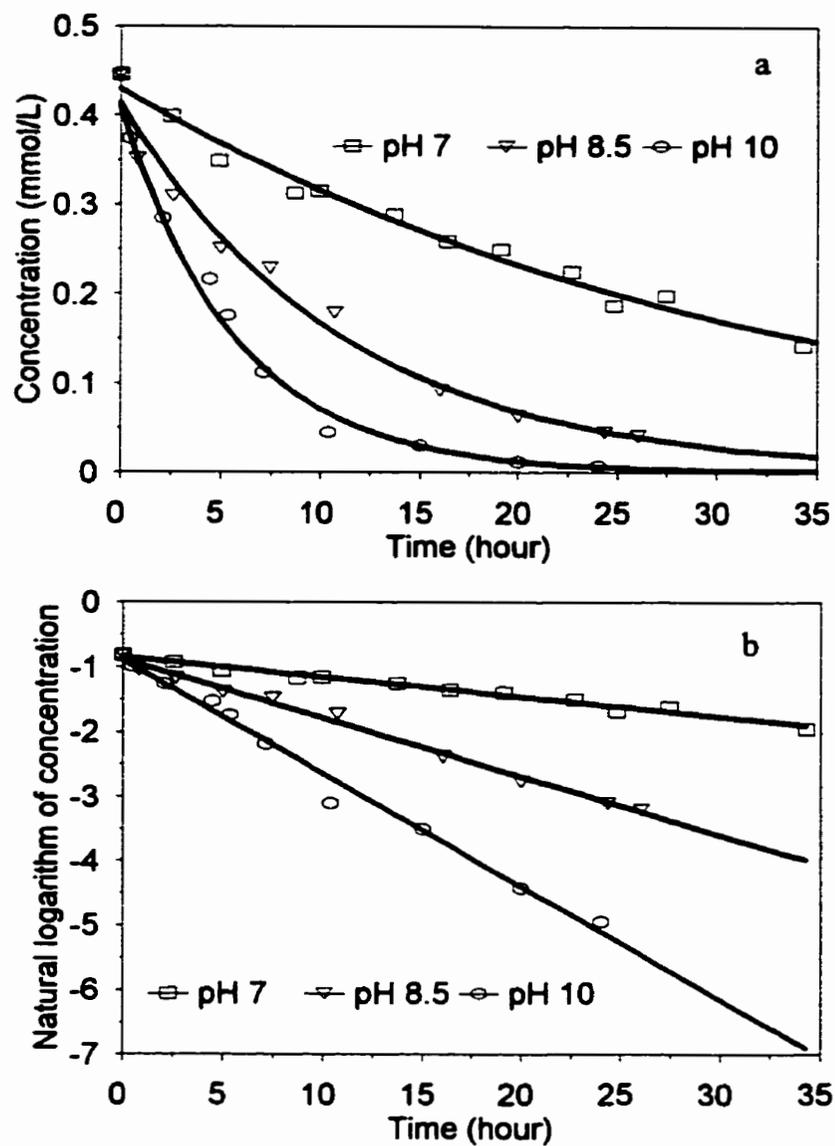
Fig.3.5. Reaction rate curves of sinapic acid (0.446 mmol/L) in phosphate-boric acid
buffers of pH 7, 8.5 and 10. (a) normal scale of concentration and (b) natural
logarithm scale of concentration. Actual measurements are reported by markers,
lines represent calculated values.

values coincided well with the determined values (Fig.3.5a). The plots of the natural logarithms of sinapic acid concentration as a function of time gave straight lines for the three reactions (Fig.3.5b), indicating that the reactions were first order. Reaction order in ammonium bicarbonate buffers were the same as those in phosphate-boric acid buffers.

C. Color changes in relation to structural changes

1. Color intensity, hunter L a b values

The effects of time on the Hunter L a b values of the reaction mixtures (phosphate-boric acid buffers) at pH 7, 8.5 and 10 are shown in Fig.3.6a-c. The color of the reaction mixture at pH 7 was initially almost unchanged in comparison with the control (sinapic acid in deionized water with a natural pH of 4.3). However, this color intensity steadily increased with time as seen by a decrease in the L value and an increase in the magnitude of both a and b values (Fig.3.6a). For reactions at pH 8.5 and 10 (Fig. 3.6b-c), yellow intensity (b) was higher than that of the control at the beginning of the reaction. This color increased during the initial stage of the reaction by 18 hours and then slightly decreased as the reaction reached 28 hours. It increased again by 169 hours, when 2,6-dimethoxy-p-benzoquinone and 6-hydroxy-5,7-diemthoxy-2-naphthoic acid were found to be present. The initial high yellow intensity of the solution could be attributed to the absorption of the sinapic acid phenolate anion (anion II), as seen from the UV spectra (Fig.3.4a). This color increased and then faded slightly during the course of the conversion to thomasidioic acid from sinapic acid. According to the HPLC results, 92% (pH 8.5) and nearly 100% (pH 10) of sinapic acid was converted
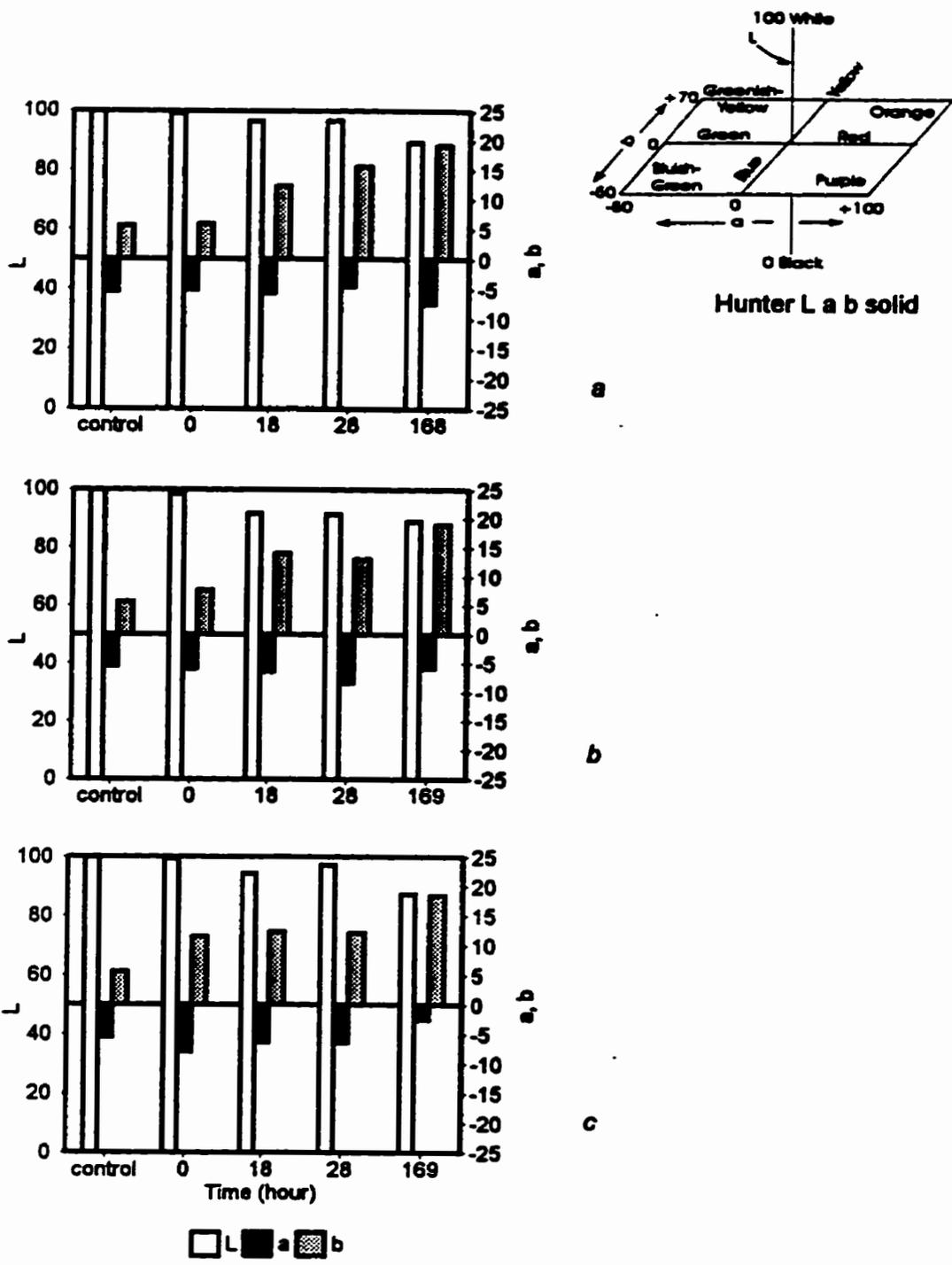
Fig.3.6a-c. Effect of reaction time on the Hunter L a b values of a 100 μg/mL sinapic acid solution in phosphate-boric buffers of (a) pH 7, (b) pH 8.5 and (c) pH 10. Control was sinapic acid in H_2O with a natural pH of 4.3.

to thomasidioic acid within 28 hours. Therefore, yellow intensity at this time should reflect the color of the phenolate anions of thomasidioic acid. Moreover, the conversion to thomasidioic acid from sinapic acid may involve the formation of some intermediates, which may also have an effect on the color properties of the system. It seemed apparent that at least one intermediate between sinapic acid and thomasidioic acid must account for some color. Since 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid were found together with an increased color darkening for the system for the late period of reaction, these substances could be major color contributors to the system.

Color profiles in ammonium bicarbonate buffers at pH 8.5 and 10 (Fig.3.7a-b) were similar to those found in phosphate-boric acid buffers, but the color changes were more remarkable. The initial yellow of the sinapic acid anions was followed by an increase and then a decrease, concurrent with the conversion of sinapic acid to thomasidioic acid, which was nearly complete after two hours at pH 10, and three hours at pH 8.5. This was followed by a steady increase in color intensity, when the 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-diemthoxy-2-naphthoic acid were found to be present after 72 hours.

2. Color property of sinapic acid and thomasidioic acid in basic conditions

In order to evaluate the color properties of sinapic acid and thomasidioic acid, the colors of the two compounds in phosphate-boric acid buffers were determined with visible light transmittance spectra. Both compounds showed little color at pH 7 and showed a remarkable decrease in percentage transmittance as pH increased from 7 to 10. These agreed
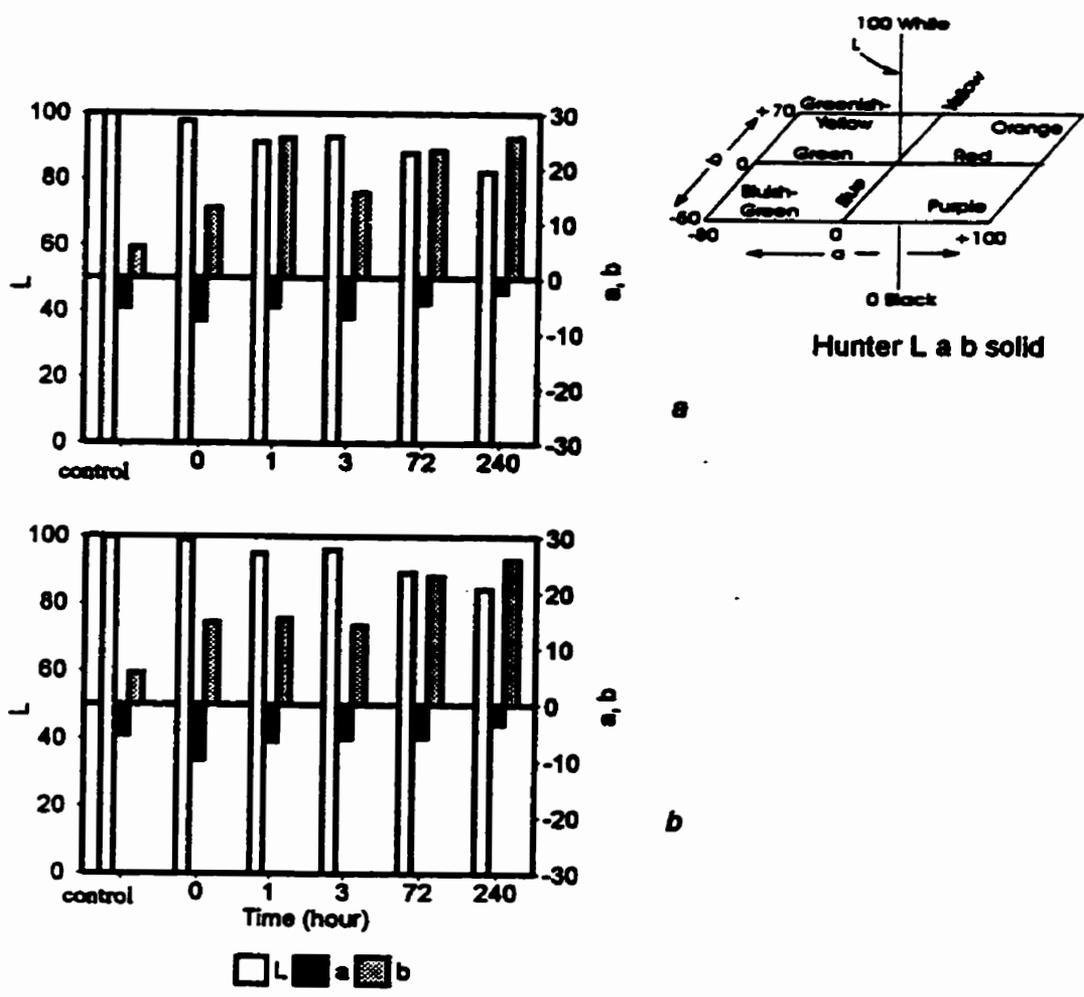
Fig.3.7a-b. Effect of reaction time on the Hunter L a b values of a 100 μg/mL sinapic acid solution in ammonium bicarbonate buffers of (A) pH 8.5 and (b) pH 10. Control was sinapic acid in H_2O with a natural pH of 4.3.

with the UV spectra where a large absorption band was found for both compounds in the visible region. However, the transmittance decreases for thomasidioic acid at pH 8.5 and 10 were less pronounced than those for sinapic acid. This indicated that thomasidioic acid was less colored than sinapic acid at pH 8.5 and 10. However, the colors of the oxidized solutions of sinapic acid after 28 hours (Fig.6-7) were not always lower than, but about equal to, that of the solutions at zero time. A comparison between transmittance spectra of equal weight concentrations of sinapic acid and thomasidioic acid at different pHs is shown in Fig.3.8a-b.

As the color properties of sinapic acid and thomasidioic acid are pH dependent, the color determined at basic conditions will be different from those determined at neutral or acidic conditions. Similarly, during protein isolation from canola meal with basic extraction and acidic precipitation, the colored sinapic or thomasidioic anions at basic conditions will change to colorless during acidic precipitation provided no other interactions between protein and phenolics occur. However, the secondary oxidation products such as the 2,6-dimethoxy-p-benzoquinone should remain as colored substances if these substances are present.

3. Spectral property of 6-hydroxy-5,7-dimethoxy-2-naphthoic acid and 2,6-dimethoxy-p-benzoquinone

UV and light transmittance spectra of the 6-hydroxy-5,7-dimethoxy-2-naphthoic acid at different pHs are shown in Fig.3.9a-b. At pH 7, the 2-naphthoic acid showed two major peaks at around 250 and 300 nm (Fig.3.9a). Higher pH caused a significant shift of the peaks towards higher wavelengths (around 255 and 330 at pH 10). However, these shifts, in fact,
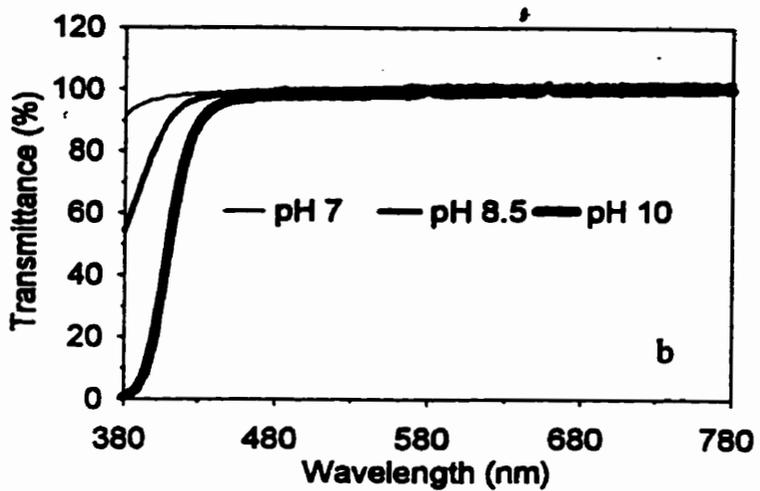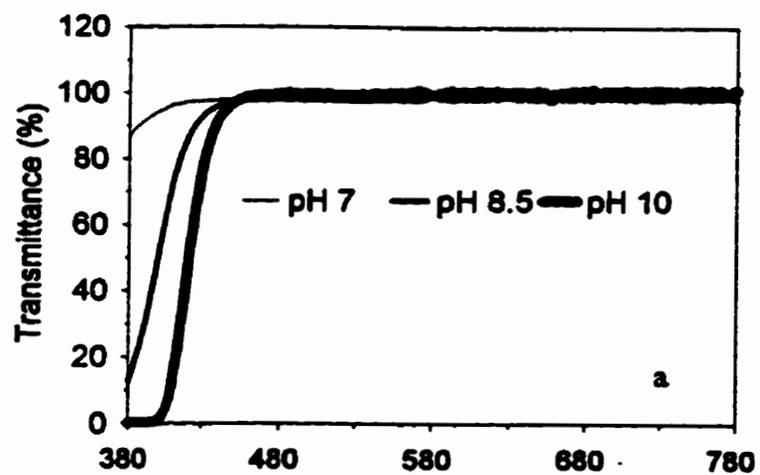
Fig.3.8a-b. Transmittance spectra of a 100 μg/mL phosphate-boric buffer solution of pH 7, 8.5 and 10 (a) sinapic acid and (b) thomasidioic acid.
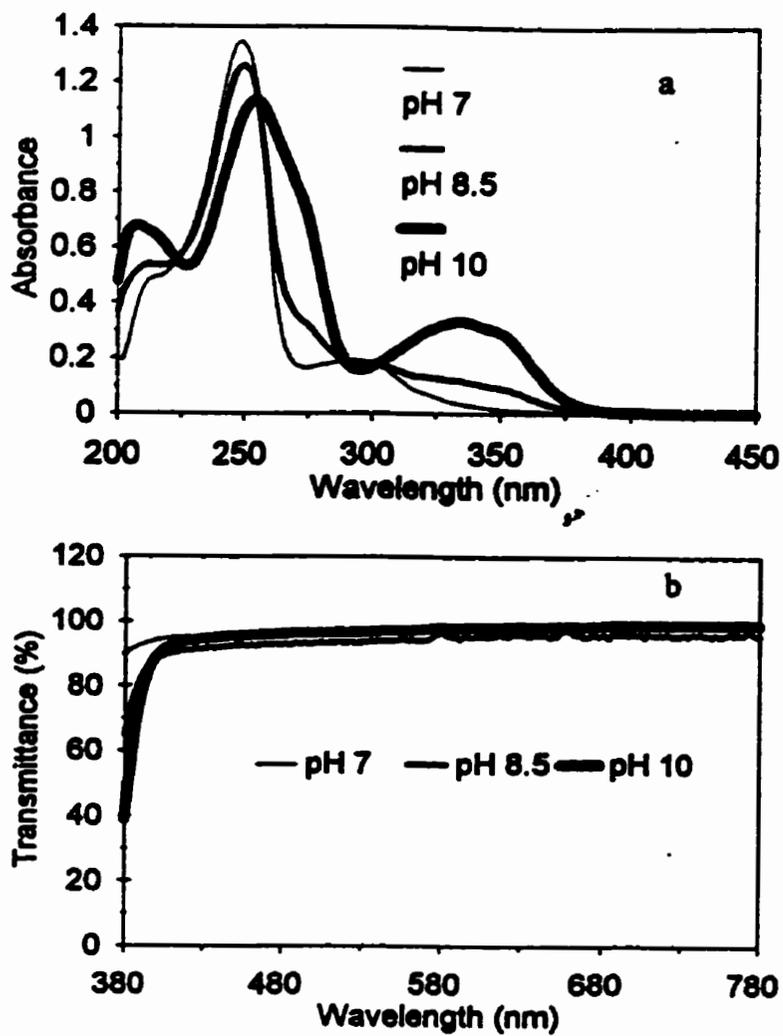
Fig.3.9a-b. UV (diluted 10 times) absorbance (a) and visual light transmittance (b) spectra of a 100 μg/mL 6-hydroxy-5, 7-dimethoxy-2-naphthoic acid.

did not cause a large change in the visual light transmittance spectra (Fig.3.9b). At pH 7 the 2-naphthoic acid showed little color. The color intensity only had a slight increase as pH increased from 7 to 10, indicating it not to be a significant color contributor even under basic conditions. The 2-naphthoic acid has been reported as pale tan needles (Hostettler and Seikel, 1969).

A comparison of UV and light transmittance spectra of the 2,6-dimethoxy-p-benzoquinone is shown in Fig.3.10a-b. In the UV spectra (Fig.3.10a), 2,6-dimethoxy-p-benzoquinone showed a maximum absorbance at 280 nm in contrast to the two maxima of sinapic acid at 230 and 325 nm. While sinapic acid showed no absorbance at wavelengths above 380 nm, the p-benzoquinone showed some absorbance over this region, indicative of a colored substance. In the light transmittance spectra (Fig.3.10b), 2,6-dimethoxy-p-benzoquinone showed a noticeable low transmittance in the wavelength range from 380 to 480. This is consistent with the yellow appearance of that substance itself (Hostettler and Seikel, 1969). This also indicated that p-benzoquinone was a strong contributor to the color of the system. It was noted from HPLC analysis shown in Fig.3.3b that there were other undetermined colored substances produced during the reaction. These substances, however, may not necessarily correspond to the substances in the basic solutions since the HPLC was done at pH 4.7. For instance, the colored thomasodioic anion in the basic solutions would not show up under this condition.
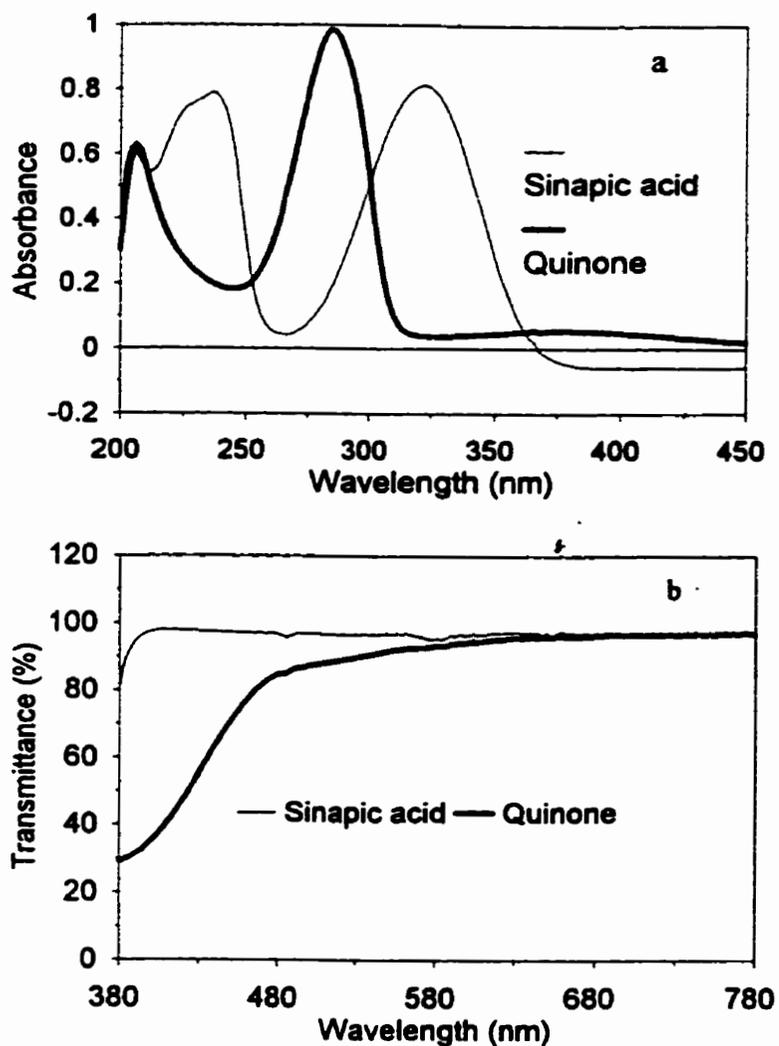
Fig.3.10a-b. UV (diluted 10 - 20 times) absorbance (a) and visual light transmittance (b) spectra of a 100 μg/mL 2, 6-dimethoxy-*p*-benzoquinone and a 100 μg/mL sinapic acid (all in methanol).

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D. Mechanisms of the reactions

Results from this research showed that after 28 hours there was 92% (pH 8.5), and nearly 100% (pH 10) disappearance of sinapic acid in phosphate-boric acid buffers (100 μg/mL). Bathochromic shifts at the beginning of the reaction were caused by the formation of phenolate anions, which could be responsible for the initial colors of the mixtures (Ribereau-Gayon, 1972; Harborne, 1964; Smyk and Drabent, 1989). The color increased and then slightly decreased in the first 28 hours at pH 8.5 and pH 10 during the course of the conversion of sinapic acid to thomasidioic acid. Similar color changes were demonstrated in reactions in ammonium bicarbonate buffers.

Lee (1997) determined that the dimerization of sinapyl radicals was the most likely pathway for oxidation of sinapic acid. The dimerization product, a bis-quinone methide, would then partially tautomerize to form a mono-quinone methide and then cyclize to form thomasidioic acid. The mechanism for the conversion of sinapic acid to the 2,6-dimethoxy-p-benzoquinone and the 6-hydroxy-5,7-dimethoxy-2-naphthoic acid was determined to involve the secondary oxidation of the first formed thomasidioic acid (Charlton and Lee, 1997). If this is the case, then the two intermediates, the bis-quinone methide and the mono-quinone methide may also affect the color profile of the system. The intermediates may account for the color intensity observed during conversion of sinapic acid to thomasidioic acid.

With time, thomasidioic acid gradually oxidized to form the p-benzoquinone and the 2-naphthoic acid, which in turn increased the yellow intensity with the p-benzoquinone being the major color contributor. This is also the case for the reaction at pH 7. The steady increase

in color intensity is consistent with the air oxidation process of sinapic acid to form thomasidioic acid and further oxidation to the *p*-benzoquinone and the 2-naphthoic acid. The darkening of color at pH 7 is particularly noteworthy since it had a neutral pH value and there was no significant sign of color changes at the onset of the reaction, unlike the more basic pH conditions, which produced immediate yellow colors.

A reaction scheme showing the conversion of sinapic acid to thomasidioic acid, and further oxidation to 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid is given in Fig.3.11.

In conclusion, alkaline air oxidation of sinapic acid to form thomasidioic acid fit well with a first order rate equation. Reaction rate constants (k) were determined to be 8.54×10^{-6}, 2.51×10^{-5} and 4.87×10^{-5} s^{-1} in phosphate-boric acid buffers at pHs of 7, 8.5 and 10, respectively. Similar reactions in ammonium bicarbonate at pH 8.5 and 10 were found to be more than ten times faster. Generally, color intensity tended to increase as reaction proceeded. 2,6-Dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid were associated with a high color intensity. The colored nature of 2,6-dimethoxy-*p*-benzoquinone and the presence of this substance in the reaction mixture indicated it to be a strong color contributor. However, the time required for the development of the colored compounds was much longer than that required for the protein isolation. Therefore, it is doubtful whether those compounds will be present during protein isolation.
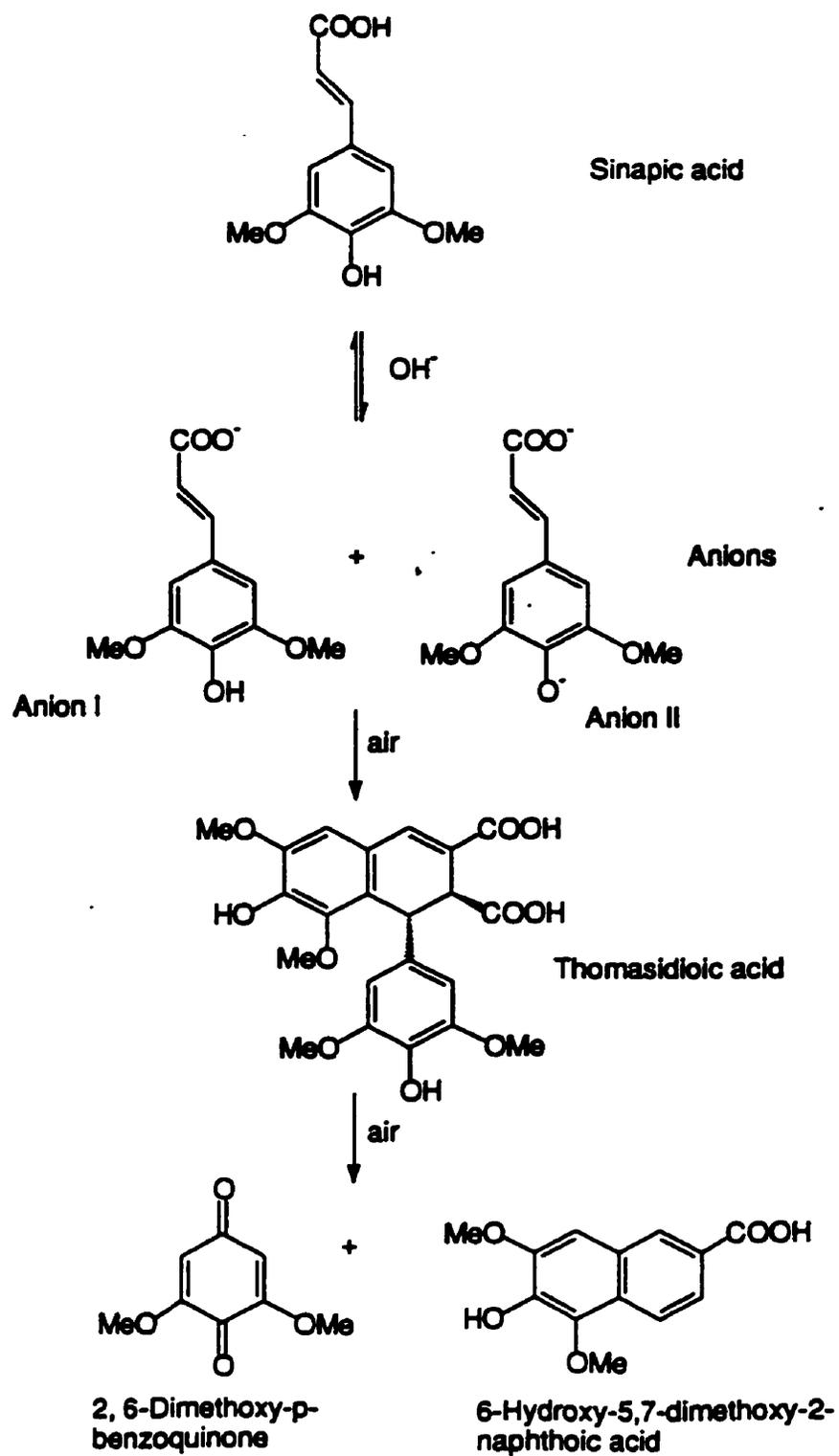
Fig.3.11. Alkali induced air oxidation of sinapic acid.

CHAPTER IV. COLOR AND STRUCTURAL CHANGES OF SINAPINE BISULFATE DURING ALKALINE HYDROLYSIS AND AIR OXIDATION

ABSTRACT

Structural changes of sinapine were induced by alkaline hydrolysis and air oxidation and followed by spectral and high performance liquid chromatographic (HPLC) analysis. Color properties of aqueous sinapine solutions were determined by taking the transmittance spectra, calculating the Commission Internationale de l'Eclairage (CIE) 1931 tristimulus values and converting to Hunter L a b values. Reaction rate constants for sinapine were generated by a kinetic study based on the quantitative results from HPLC analysis. These reactions were first order with respect to sinapine and fit the appropriate equation with a coefficient of $R^2 > 0.97$. Sinapine was first hydrolyzed to sinapic acid, which then converted to thomasidioic acid. Reaction rate constants for sinapine were 9.70×10^{-7}, 4.58×10^{-6} and 8.85×10^{-1} s^{-1} in phosphate-boric acid buffers of pH 7, 8.5 and 10, respectively. Results of spectral analyses showed that the colors of all the systems at pH 7, 8.5 and 10 darkened as reaction time increased.

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INTRODUCTION

Among the problems associated with canola protein isolation, such as dark color, low yield and the presence of antinutritional substances (glucosinolates, phytates and phenolics) (Shahidi, 1990; Shahidi and Naczk, 1992), dark appearance of the canola protein products is one of the major problems that hinders the use of canola meal, an abundant oilseed by-product across the world (Youngs, 1991; Keshavarz, et al., 1977). A dark meal results immediately following the hexane extraction and desolventizing in oil mills (Shahidi, 1990; Youngs, 1991). Further processing of the meal results in a protein concentrate or isolate whose color is usually not improved. For example, dark appearance of a protein isolate has been reported by the method of isoelectric precipitation (Keshavarz, et al., 1977) and by a protein micellar mass procedure (Ismond and Welsh, 1992; Gruener, 1996). This dark appearance was particularly evidenced with the products produced using the basic extraction with isoelectric precipitation, where the meal is first extracted with an alkaline solution and acidified to precipitate the protein at its isoelectric point (Gillberg and Tornell 1976a; Keshavarz et al., 1977).

The occurrence of a coloration from the colorless sinapine solution when exposed to an alkali stimulus has been reported quite early (Austin and Wolff, 1968). This coloration was attributed to a bathochromic shift due to the formation of the corresponding anionic forms from neutral phenolics determined by a spectral analysis (Harborne 1964; Ribereau-Gayon,

1972). With canola protein, the presence of phenolics could result in the development of a brown coloration (Sosulski and Bakal, 1969). A procedure in which rapeseed meal was extracted with aqueous NaCl solution, followed by isoelectric precipitation has been reported to yield a light color for the protein isolate (Owen et al., 1971). Keshavarz, et al. (1977) compared the color of the product from several isolation procedures and attributed the dark color to the presence of alkali stimuli. Appelqvist (1971), however, suggested that hull pigments could be the cause of the color in these products. In general, such observations are associated with the presence of some minor components that are sensitive to processing conditions.

The role of color development has received much attention by several researchers (Shahidi and Naczk, 1992; 1995; Sosulski, 1979; Keshavarz et al., 1977). Phenolics have been considered as a predominant factor in determining the color properties of the oilseed by-products. The dark color of sunflower meal has been attributed to the presence of chlorogenic acid (Shahidi and Naczk, 1995). Phenolics also play a role in the color of the soy protein (How and Morr, 1982). Canola phenolics have been reported to have the effects not only on color but also on bitterness and nutritional properties of canola protein (Shahidi and Naczk, 1992). However, color changes resulting from structural changes in sinapine at different pHs and reaction times have not been determined. These colored changes would provide information on the color significance of sinapine for the system. In addition, reaction rate constants for the degradation of sinapine at different pH conditions have not been determined either, and these could provide useful kinetic information when considering

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protein isolation under alkaline conditions.

As part of a series of studies looking at the effect of phenolics on the color of canola protein isolate, the color and structural changes of sinapine in aqueous solutions of pH 7 - 10 were investigated. Reaction rate constants were determined at pH 7, 8.5 and 10.

MATERIALS AND METHODS

A. Sources of materials

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sinapine was isolated from *Sinapis alba* certified seed from Tilney Mustard crop as sinapine bisulfate according to the method outlined by Clandinin (1961). Sinapine bisulfate was used in place of sinapine throughout the research. Thomasidioic acid was prepared according to the procedure outlined by Ahmed et al. (1973) and Rubino et al. (1995) with some modifications. Sinapic acid (500 mg) was dissolved in 10 mL methanol. Slightly warming the mixture was necessary to get a complete dissolution of sinapic acid in the methanol. This solution was added slowly (in about 10 min) to a solution of $FeCl_3$ $(H_2O)_6$ (1.6 g) in water (40 mL) while a rapid stream of oxygen was passed through the $FeCl_3$ solution. A red-violet precipitate formed immediately once the sinapic acid solution was added. The oxygen passage continued for about five hours before the mixture was allowed to stand overnight. The mixture was filtered through a filter paper (Waterman No. 1) and the paste was suspended in water (120 mL). The mixture was filtered again and washed with water. To the paste, 10 mL H_2SO_4 was added and the mixture was vigorously shaken for 30 seconds. Then, the mixture was diluted with 50 mL water. After cooling to the room temperature, the mixture was extracted three times with ethyl acetate (20 mL). The organic phases were combined, dried with $MgSO_4$ and evaporated under vacuum to remove

the solvent. This yielded a brown solid. The solid was recrystallized from CH_2Cl_2 to yield the thomasidioic acid as a light tan powder. This thomasidioic acid was used as a standard in identifying the thomasidioic acid formed by air oxidation of sinapic acid.

Acetic acid and sodium hydroxide used for HPLC were verified ACS-grade and purchased from Fisher Scientific Co. (Nepean, ON, Canada). Other chemicals used for HPLC were HPLC-grade. All other chemicals, unless stated otherwise, were verified ACS-grade and purchased from Fisher Scientific Co.

B. Sample preparation

Phosphate-boric acid buffers were prepared according to Britton and Robinson (1931).

A sinapine bisulfate solution (200 µg/mL) was prepared with deionized water. Three samples of 4 mL each in phosphate-boric acid buffer of pH 7, 8.5 and 10, respectively, were prepared by combining 2 mL of the sinapine bisulfate solution with 2 mL of each buffer solution of different pHs so that 100 µg/mL (0.246 mmol) sinapine solutions with different pHs were obtained. These solutions were stirred in air at room temperature (22 °C). Water vapor loss was determined to be 1% per day under the test condition. This loss was ignored during the kinetic study. The pH values of these solutions were checked before each analysis was made. Only the pH value of the phosphate-boric acid solution at pH 10 had a slight deviation (decreased approximately 0.4 units per day) and was readjusted by adding sodium hydroxide. Spectral and HPLC analysis were carried out for a period of 4.5 days. A sample

size of 1 μL was used for HPLC analysis during the kinetic study whereas 20 μL was injected into the HPLC during the identification of color components in the solution of pH 10 using a 25-μL sample loop. Three samples of pH 7, 8.5 and 10 for ultraviolet spectral analysis were prepared by a 10-fold dilution of the sinapine solution (200 μg/mL) with phosphate-boric acid buffers of different pHs and stirred in air at room temperature (22 °C). For HPLC analyses of the standard sinapic acid and thomasidioic acid, these compounds were dissolved directly in the appropriate buffers just before analysis.

C. HPLC analysis

Chromatographic equipment consisted of two Waters (Milford, MA) pumps (model 501 and 510) and an automated gradient controller model 680, a Shimadzu (Kyoto, Japan) SPD-6A ultraviolet (UV) spectrophotometric detector, and a Hewlett-Packard (Avondale, PA) model HP3396II integrator. A reverse-phase C18 column (Supelcosyl, 3-μm particle size, 33x4.6 mm i.d.; Supelco, Bellefonte, PA) was used. Component A was a 0.05 M acetate buffer prepared by a 1:100 dilution of a stock pH 4.7 acetate buffer. The stock buffer was prepared by adjusting 5 M acetic acid to pH 4.7 with solid sodium hydroxide (Hagerman and Nicholson, 1982). Component A was filtered through a 0.45 μm filter. Component B was 100% HPLC-grade methanol. The column was maintained at 37 °C and run at a constant flow rate of 1.4 mL/min.

In monitoring the structural changes in sinapine and the disappearance of sinapine with time for the kinetic study, the initial elution solvent was 15% methanol and 85%

component A. A 10-min linear gradient was used to alter the solvent from 15% to 100% methanol. This solvent composition was maintained for 2 min, after which another 2-min linear gradient returned the solvent to its original composition.

In identifying any possible colored substances using a 400-nm detector, the initial solvent was 15% methanol and 85% component A. After a10.5-min isocratic flow, a 1.5-min linear gradient was used to change the solvent composition to 45% methanol and 55% component A. A 5-min concave gradient was used to alter the composition from 45% methanol to 100% methanol. This solvent composition was maintained for 2 min, after which a 2-min linear gradient returned the solvent to its original composition (15% methanol).

D. Kinetic study

Reaction rate constants (k) were obtained by a kinetic study based on the time dependent change in concentration of sinapine as determined from the peak areas of the HPLC chromatograms, following the same procedure as in Chapter III (Cai et al., 1999b).

E. Spectral analysis and color determination

Spectral analysis and color determination followed the same procedure as in Chapter II (Cai et al., 1999a).

RESULTS AND DISCUSSION

A. HPLC analysis

Results of HPLC analysis (Fig.4.1a-c) showed that sinapine was converted to several compounds upon alkaline hydrolysis and air oxidation at pH 7, 8.5 and 10. After 48 hours, sinapic acid was found to be present due to the hydrolysis of sinapine. An unknown compound (peak 3) was also found (Fig.4.1a). The unknown compound became pronounced after 96 hours while sinapic acid decreased (Fig.4.1a). There was only 32% sinapine converted to other compounds over the 96 hours. At pH 8.5 (Fig.4.1b), sinapic acid was detected even at zero time. After 48 hours, the peak pattern was similar to that of the solution at pH 7, except that an additional peak, identified as thomasidioic acid (peak 1), was also found. After 96 hours, the peak of sinapic acid became smaller while thomasidioic acid and the unknown (peak 3) became more pronounced. Seventy nine percent of the sinapine was converted to other compounds after 96 hours. At pH 10, sinapic acid was found to be present at zero time (Fig.4.1c). After 48 hours, a peak identified to be thomasidioic acid and the unknown peak (peak 3) were also found. After 96 hours, the peak of sinapic acid became smaller while several unknown peaks (including peak 3) appeared. Ninety six percent of the sinapine was converted to other compounds after 96 hours. Under all three pH conditions, sinapine content decreased with time as sinapine was converted to sinapic acid, thomasidioic acid and other compounds. These HPLC chromatograms (Fig.4.1a-c) and data from time
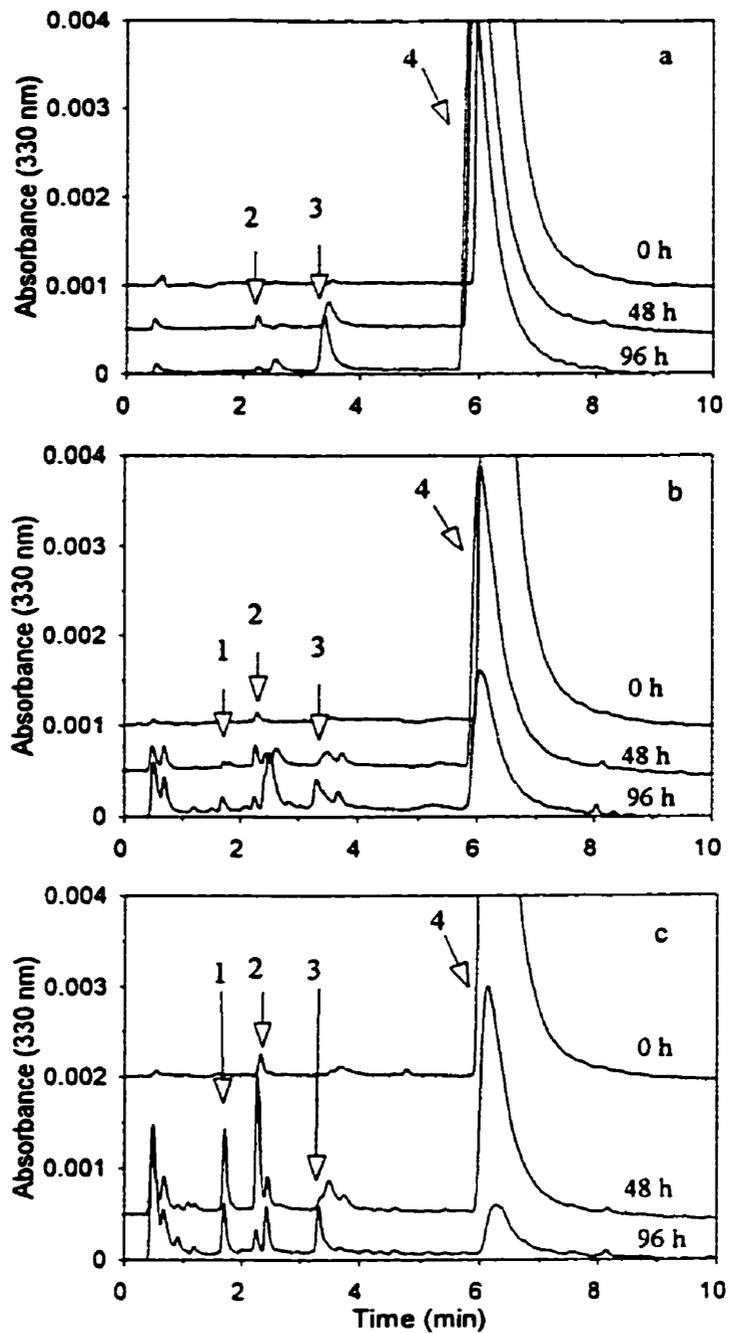
Fig.4.1a-c. HPLC chromatograms of the sinapine reaction products with reactions
conducted in phosphate-boric acid buffer of (a) pH7, (b) pH 8.5 and (c) pH 10. 1.
Thomasidioic acid, 2. sinapic acid, 3. unidentified, and 4. Sinapine. Elution
gradient: linear, 0 - 10 min, 15% - 100% methanol.

intermediate to those presented also provided kinetic data for the disappearance of sinapine during alkaline hydrolysis and air oxidation.

Both sinapic acid and thomasidioic acid are colorless compounds (Ahmed et al., 1973). No colored compounds were identified in the sinapine solution. Nevertheless, the color of the solution was found to darken throughout the reaction. The formation of sinapic acid and thomasidioic acid should not cause the darkening of the system since both compounds were found to be less colored than sinapine at equal weight and at the same pH value (Cai et al, 1999b; Appendix 3.7a-f, 4.5a-c). Therefore, examination of the color of these new compounds in the system was warranted. The HPLC chromatograms of the sinapine solution at pH 10 after 96 h for both 330 and 400 nm are shown in Fig.4.2a-b. A strong absorbance at 400 nm would be an indicative of a yellow substance. Of those compounds detected at 330 nm (Fig.4.2a), none showed strong absorbance at 400 nm (Fig.4.2b). Peak 3 had a weak absorbance at 400 nm, possibly indicating a compound with some color. A colored substance was eluted near the void volume (Fig.4.2b). However, this compound was not identified.

B. Kinetic study:

It was found that the reaction rate data fit a first order equation better than a second order equation. The correlation coefficients (R^2) for the first order equation were 0.98, 0.99 and 0.98, respectively, for the reactions in phosphate-boric acid buffers of pH 7, 8.5 and 10, whereas R^2 for the second order equation were 0.91, 0.93 and 0.71, respectively. Therefore, the first order equations were used to calculate the reaction rate constant (k) values and the
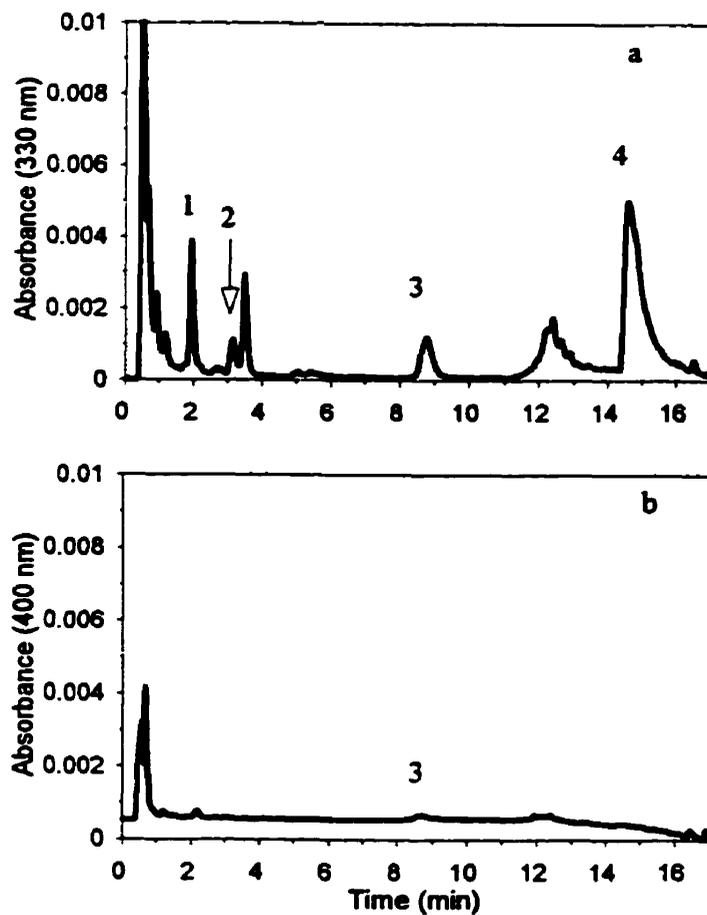
Fig.4.2a-b. HPLC chromatograms of the sinapine reaction products in phosphate-boric acid buffer of pH 10 after 96 hours at (a) 330 nm and (b) 400 nm. 1. Thomasidioic acid, 2. sinapic acid, 3. unidentified, and 4. Sinapine. Elution gradient: isocratic, 0 - 10 min, 15% methanol; linear 10 - 11.5 min, 15% - 45% methanol; concave (Fig.5.1), 11.5 - 16.5 min 45% - 100% methanol.

theoretical concentrations of sinapine for the reaction curves.

The reaction equations, reaction constants (k), half lives ($t_{1/2}$) and correlation coefficients (R^2) are summarized in Table 4.1. The reaction rate constants were 9.70×10^{-7}, 4.58×10^{-6} and 8.85×10^{-6} s^{-1}, respectively, for reactions at pH 7, 8.5 and 10. The reaction at pH 10 was about twice as fast as the reaction at pH 8.5 and about nine times as fast as the reaction at pH 7. These differences can also be seen from the half lives ($t_{1/2}$) of the reactions, which were 198.28, 42.03 and 21.74 hours for pH 7, 8.5 and 10, respectively.

Table 4.1. Reaction equations, reaction rate constants (k), half lives ($t_{1/2}$) and correlation coefficients (R^2) for reactions of sinapine aqueous solutions at pH 7, 8.5 and 10.

pH	Equation	k (s^{-1})	$t_{1/2}$ (h) [a]	R^2
pH 7 :	$\ln(c_o/c) = 0.030 + 9.7 \times 10^{-7} t$	9.7×10^{-7}	198.28	0.98
pH 8.5:	$\ln(c_o/c) = -0.001 + 4.58 \times 10^{-6} t$	4.58×10^{-6}	42.03	0.99
PH 10:	$\ln(c_o/c) = -0.010 + 8.85 \times 10^{-6} t$	8.85×10^{-6}	21.74	0.98

[a] The half life ($t_{1/2}$) for the reactions was found as:

$t_{1/2} = 0.693/k$

where $t_{1/2}$ is the half life, k the reaction rate constant.

Linear and logarithmic plots of sinapine concentrations and the corresponding plots generated from the best fit rate constants are given in Fig.4.3a-b. In general, the theoretical values coincided well with the determined values (Fig.4.3a). The plots of the natural
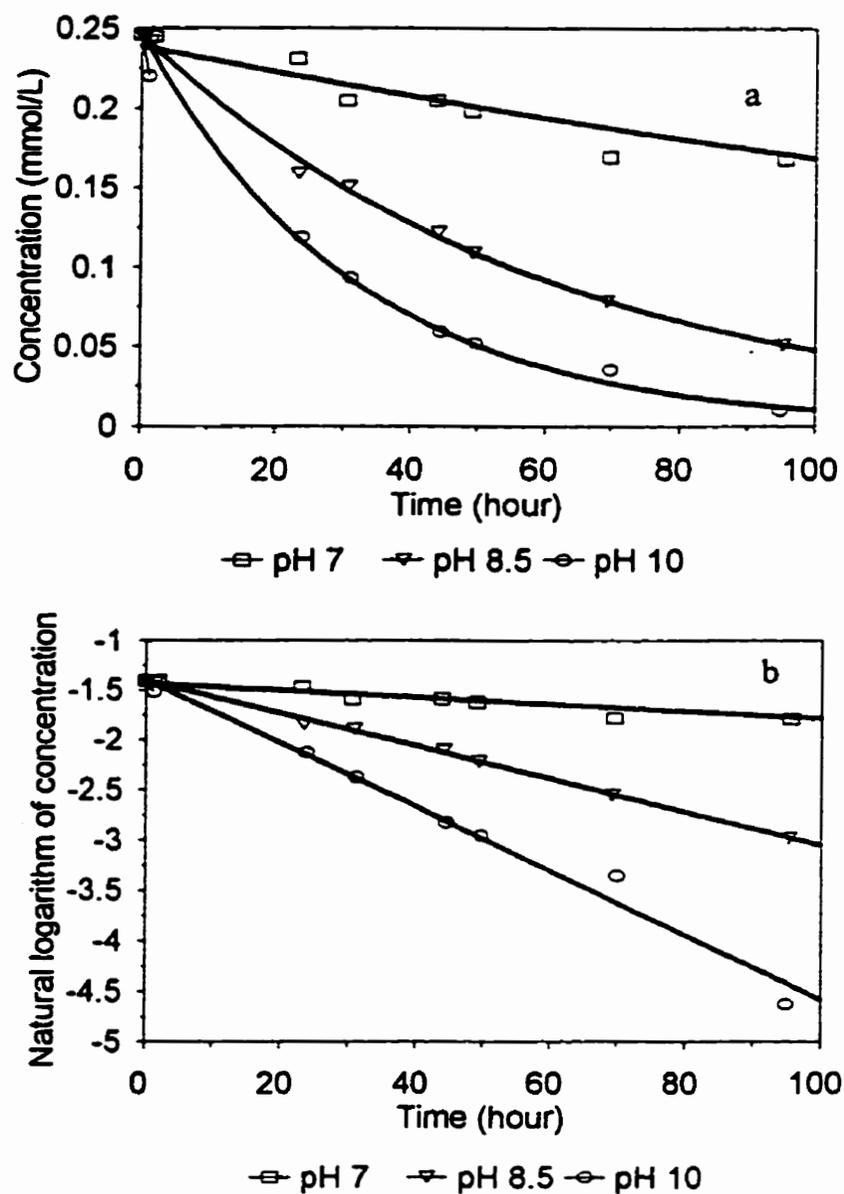
Fig.4.3. Reaction rate curves of sinapine (0.246 mmol/L) in phosphate-boric acid buffers of pH 7, 8.5 and 10. (a) normal scale of concentration and (b) natural logarithm scale of concentration. Actual measurements are reported by markers, lines represent calculated values.

logarithms of sinapine concentration as a function of time gave straight lines for the three

reactions, indicating the reactions to be first order. The reaction rate curves in Fig.4.3a-b and

resulting equations (Table 4.1) can be used to predict changes in sinapine concentration under

these conditions. Therefore, it will be useful in evaluating the possible structural changes of

sinapine when present during protein isolation from canola/rapeseed meal.

C. UV and visual spectrum

The UV spectra of 20 µg/mL sinapine solutions after alkaline hydrolysis and air

oxidation for 0, 24 and 108 hours are shown in Fig.4.4.1a-c. At zero time (Fig.4.4.1a),

increasing pH caused a strong bathochromic shift, the shift of the maximum absorbance

toward higher wavelengths, relative to the spectrum of the control (sinapine in deionized

water having a natural pH of 4.3). These shifts should be responsible for the initial color

properties of the solutions. The shift would also be expected to be similar at 24 hours

(Fig.4.4.1b) and 108 hours (Fig.4.4.1c). However, as time increased, sinapine concentration

decreased and the spectra no longer represented pure sinapine. The effect of alkaline

conditions on the absorption properties of phenolic compounds has been reported by several

researchers (Ribereau-Gayon, 1972; Harborne, 1964). Large bathochromic shifts were noted

in most cases, and an increase in the intensity of the absorption bands was also observed

(Harborne, 1964). These previous observations agree with the results of this research. The

changes in absorbance with time can be better seen by plotting the data for each pH as a

separate graph (Fig.4.4.2a-c). As time increased from zero to 108 hours, the absorbance of
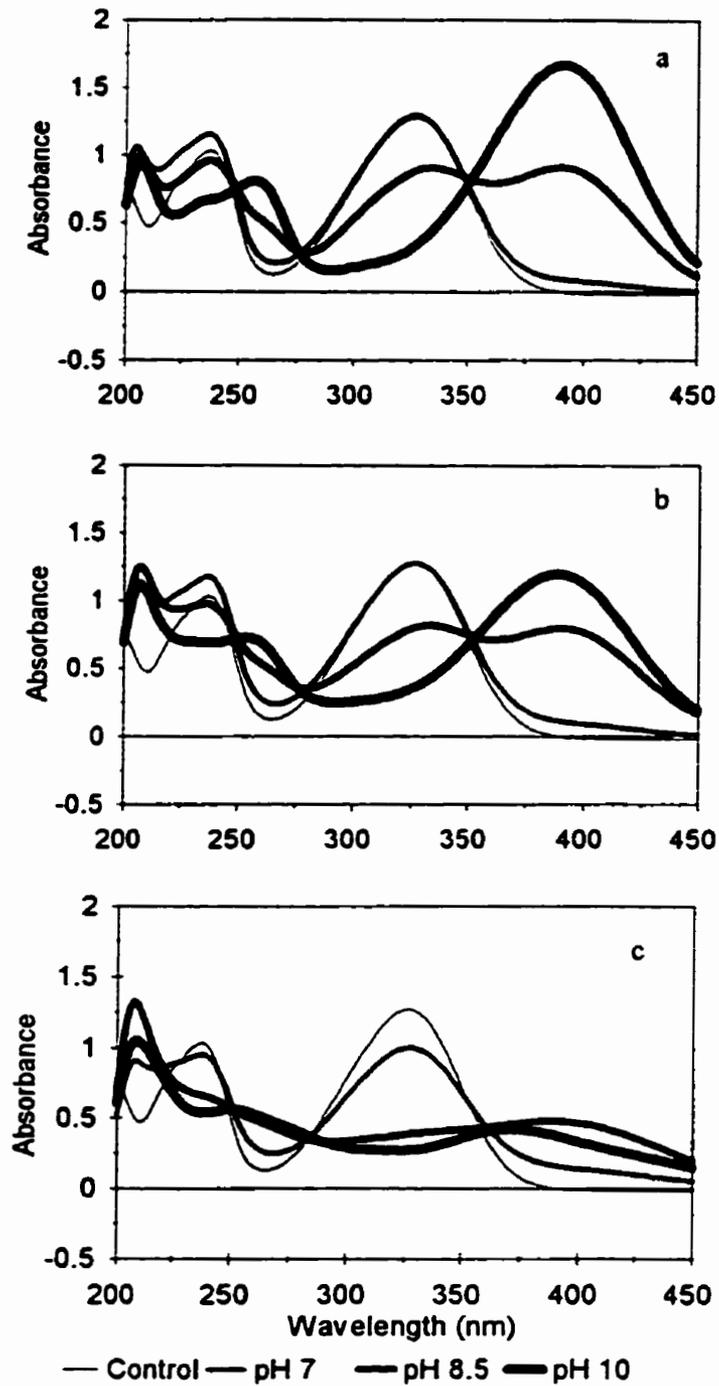
Fig.4.4.1a-c. UV spectra of a 20 μg/mL sinapine in phosphate-boric acid buffer solution of pH 7, 8.5 and 10. (a) sinapine after 0 hour, (b) sinapine after 24 hours and (c) sinapine after 108 hours. Control was sinapine in deionized H₂O with a natural pH of 4.3.
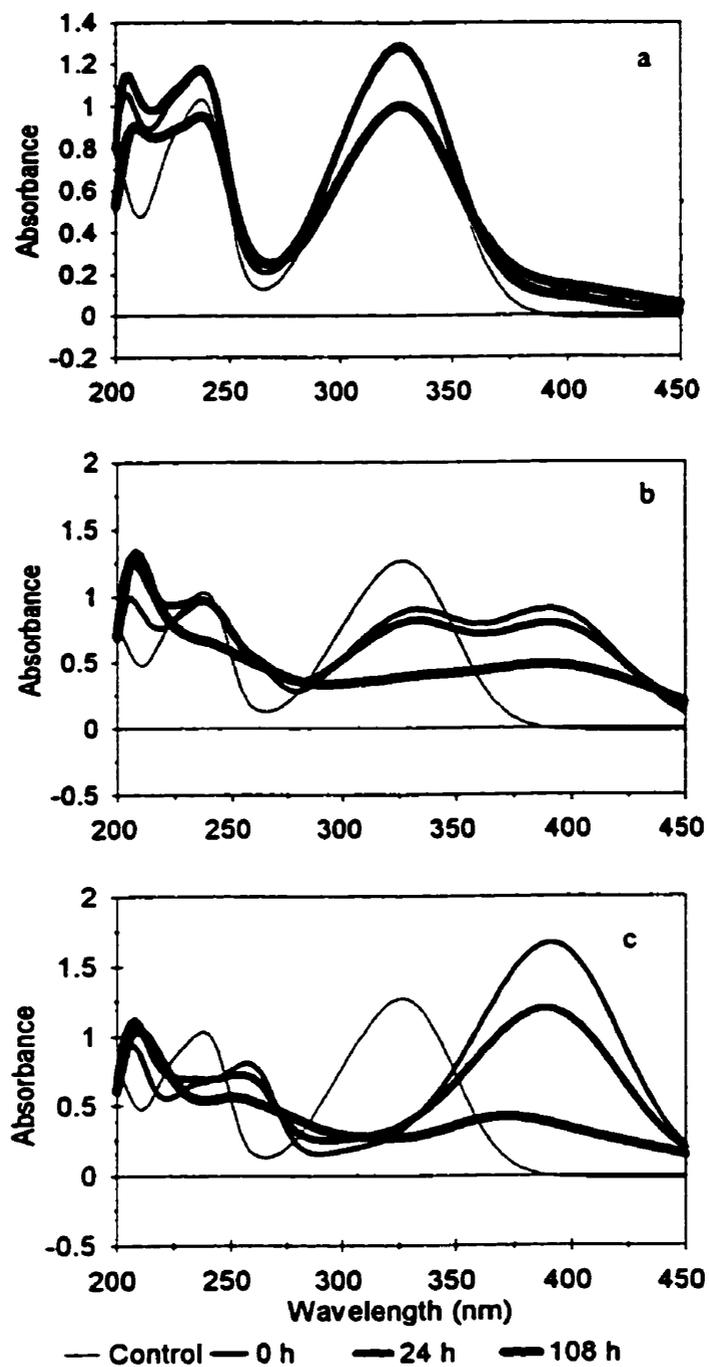
Fig.4.4.2a-c. UV spectra of a 20 µg/mL sinapine in phosphate-boric acid buffer solution after reaction for 0, 24 and 108 hours. (a) pH 7, (b) pH 8.5 and (c) pH 10. Control was sinapine in deionized H_2O with a natural pH of 4.3.

both the neutral and anionic forms of sinapine (330 and 390 nm) decreased with time. The absorbance of sinapine at pH 7 decreased at 330 nm (neutral form), that of sinapine at pH 10 decreased at 390 nm (alkaline form), while the absorbance of sinapine at pH 8.5 decreased at both the neutral (330 nm) and alkaline (390 nm) absorbance wavelengths. The decrease of absorbance with time is greater at pH 10 than at the other pH values. This agreed with the results of the kinetic study, where the reaction at pH 10 had the highest reaction rate constant, followed by the reactions at pH 8.5 and pH 7.

The results of the light transmittance spectra for 100 μg/mL sinapine solutions of pH 7, pH 8.5 and pH 10 and the control at reaction time of 0, 24 and 108 hours are given in Fig.4.5a-c. Transmittance was low in the blue light region (around 400 nm) at zero time for solutions at pH 7, 8.5 and 10. This would indicate that the solutions were yellow in color. Regardless of the reaction time, the transmittance decreased as pH increased from 7 to 10. In fact, The solution of pH 7 only had a small increase in absorbance in the blue light region from zero to 108 hours. Therefore, the color change of this solution was relatively small. After 24 hours, the solution of pH 8.5 had a new absorbance band in the green light region (around 520 nm) while the solution of pH 10 had a new band of absorbance in the red light region (around 650 nm). This should indicate a red component for the former while a green component for the latter. After 108 hours, both solutions of pH 8.5 and 10 had an absorption band in the region from 500 to 700 nm. Also, by comparing the three figures (Fig 4.5a-c), it can be seen that the transmittance of the solutions decreased with time. This may reflect a decrease in the whiteness of the solutions.
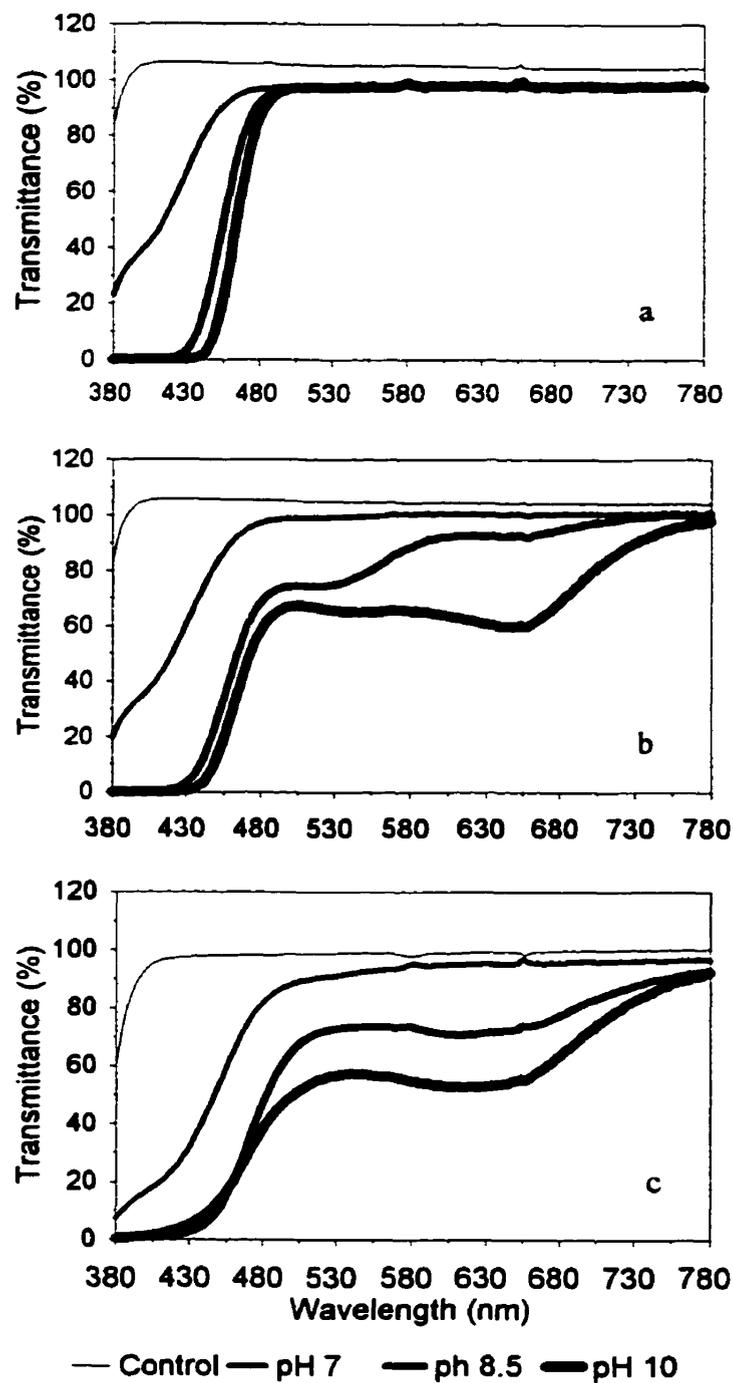
Fig.4.5a-c. Light transmittance spectra of a 100 μg/mL sinapine in phosphate-boric acid buffer solution of pH 7, 8.5 and 10. (a) sinapine after 0 hour, (b) sinapine after 24 hours and (c) sinapine after 108 hours. Control was sinapine in deionized H_2O with a natural pH of 4.3.

D. Color intensity, Hunter L a b values

The effect of time on the Hunter L a b values of the reaction solutions at pH 7, 8.5 and 10 are shown in Fig.4.6a-c. At pH 7, the color of the reaction solution steadily increased toward greenish yellow (a value decreased and b value increased) as time increased from zero to 108 hours. Darkness of the solution steadily increased (L value decreased). At pH 8.5, the b value was considerably higher than for the control, indicating an increase in yellow component. The a value decreased upon pH adjustment (zero time), increased by 24 hours and then decreased again by 108 hours. For the reaction at pH 10, the initial high greenish yellow that resulted from the pH adjustment (a = -26.22, b = 46.62) decreased as time increased to 108 hours (a = -19.24, b = 33.99), while the solution become darker (L value decreased from 97.92 to 73.36). In all cases, the colors of all the solutions tested were darker than the control, which was sinapine in deionized water with a pH of 4.3. The color of the control stayed unchanged from zero to 108 hours.

E. Mechanisms of the reactions

Bathochromic shifts were caused by the reaction of phenolics to form corresponding phenolate anions, which were responsible for the initial color of the solutions (Harborne, 1964; Ribereau-Gayon, 1972). The reactions in which sinapine converts to sinapic acid and thomasidioic acid are summarized in Fig. 4.7. The first step of the reaction involved the hydrolysis of sinapine to sinapic acid. The sinapic acid was then oxidized to form thomasidioic acid. In the study of the conversion of sinapic acid to thomasidioic acid, Lee (1997)
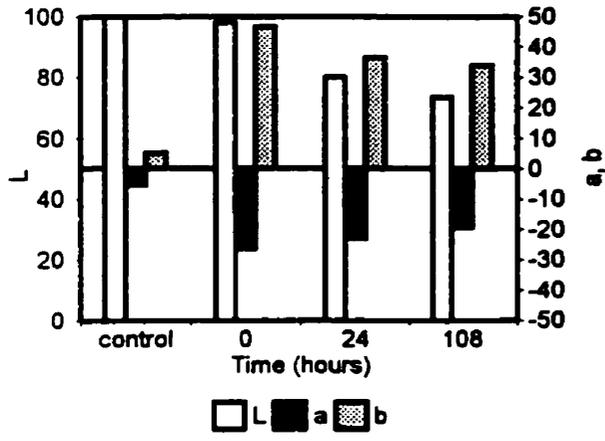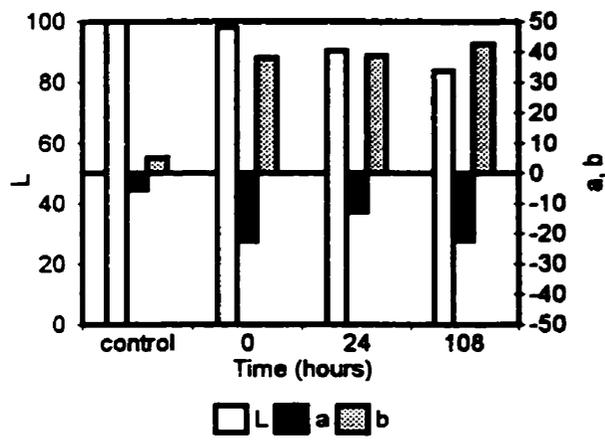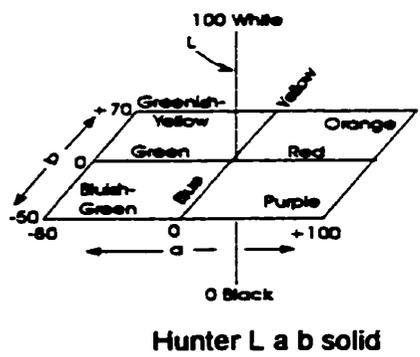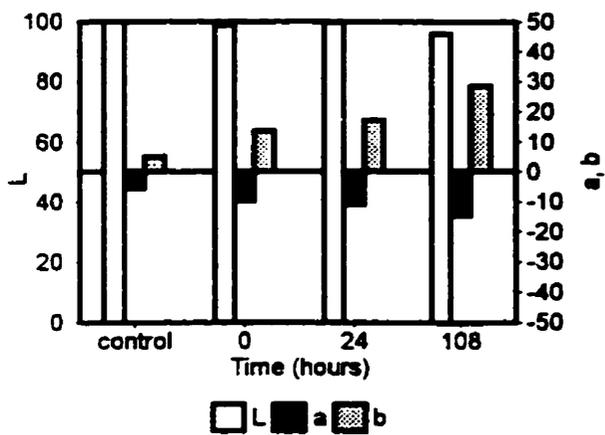
Fig.4.6a-c. Effect of reaction time on the Hunter L a b values of a 100 µg/mL sinapine in phosphate-boric buffers of (a) pH 7, (b) pH 8.5 and (c) pH 10. Control was sinapine in deionized H_2O with a natural pH of 4.3.
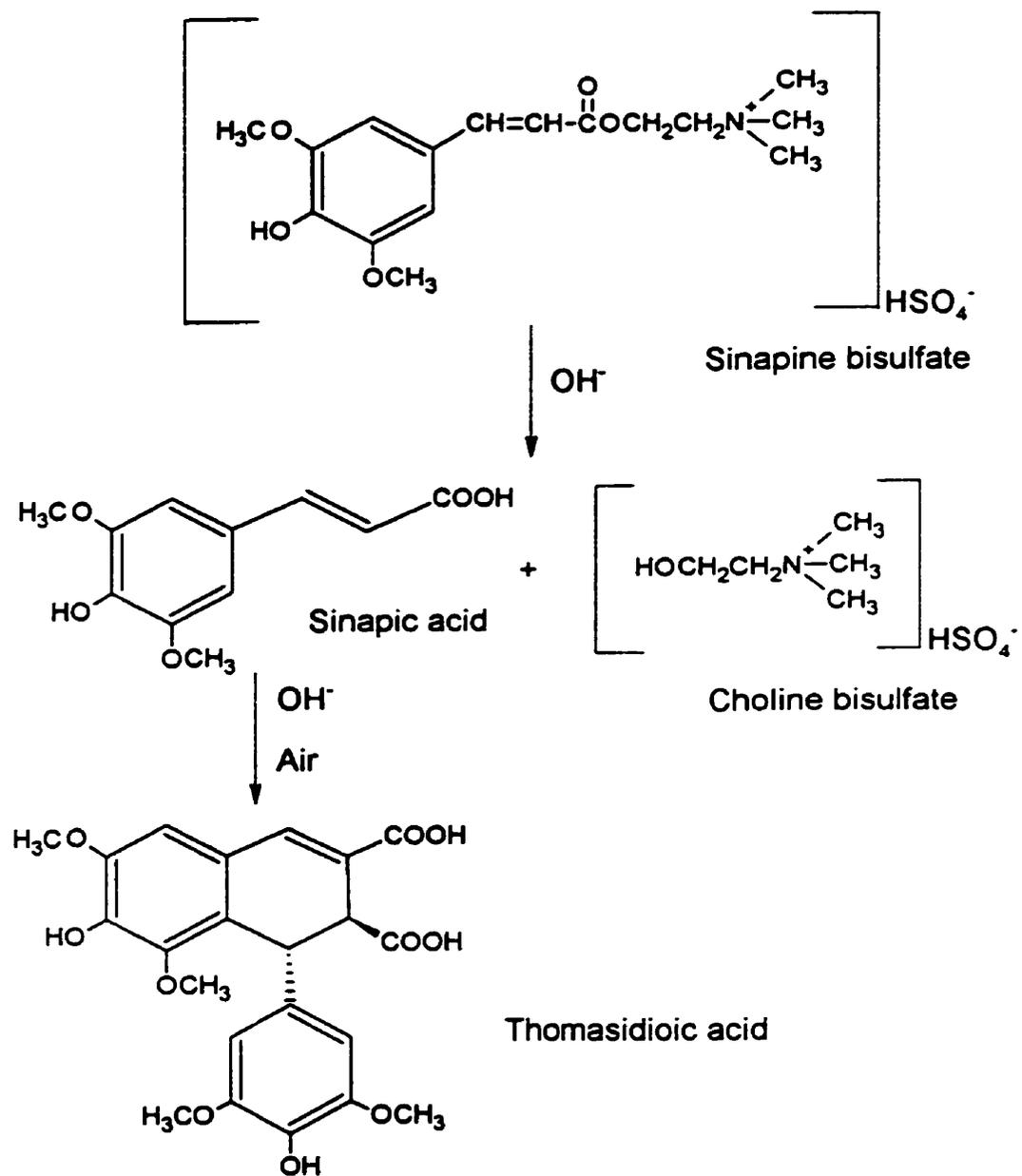
Fig.4.7. The alkaline hydrolysis and air oxidation of sinapine to form sinapic acid and thomasidioic acid.

determined that the dimerization of sinapyl radicals was the most likely pathway for oxidation of sinapic acid. The dimerization product, bis-quinone methide, would then partially tautomerize to form a mono-quinone methide and then cyclize to form thomasidioic acid.

In conclusion, the alkaline hydrolysis and air oxidation of sinapine to form sinapic acid fit well with a first order equation. Reaction constants (k) were determined to be 9.7×10^{-7}, 4.58×10^{-6} and 8.85×10^{-6} s^{-1} in phosphate-boric acid buffers at pHs of 7, 8.5 and 10, respectively. Bathochromic shifts of sinapine, caused by the formation of phenolic anions, were responsible for the initial greenish yellow color. Under all three pH conditions, colors of solutions darkened with time. Greenish yellow intensified at pH 7 and pH 8.5 with time. The greenish yellow in the solution of pH 10 decreased with time, but the low L value indicated the color moved toward the dark-brown side.

CHAPTER V. A RAPID HPLC METHOD FOR THE DETERMINATION OF SINAPINE AND SINAPIC ACID IN CANOLA SEED AND MEAL

ABSTRACT

Conventional high performance liquid chromatographic (HPLC) methods normally require purification steps for sinapine determination when applied to canola extracts. An HPLC method with no purification steps has been developed to separate sinapine and sinapic acid from other phenolics in canola seed and meal in a single run. The separation was achieved with a reverse-phase C18 column. Refluxing with 100% methanol for 20 min was selected as the best extraction method out of four different conditions tested using HPLC analysis and Folin-Ciocalteu's reagent assessment. A 10-min isocratic/linear/concave gradient and a 15-min isocratic/linear gradient were selected as the best gradients from several gradients tested. Peak identities of sinapine and sinapic acid were verified with ion-exchange separation followed by HPLC analysis. The method was calibrated using a sinapine bisulfate standard and a sinapic acid standard, with the correlation coefficient (R^2) of the calibration curves being 0.997 and 0.999 for sinapine bisulfate and sinapic acid, respectively. The extinction coefficient of sinapine was determined to be 1.16 times that of sinapic acid at the detector wavelength (330 nm), which enabled the peaks of the two compounds to be compared on the same chromatogram. Applying this method to routine canola phenolic analysis has greatly reduced the cost by simplifying the procedures and reducing the time required for each determination.

Key Words: Canola, HPLC, phenolic determination, sinapic acid, sinapine

INTRODUCTION

Sinapine is a major phenolic in canola and rapeseed (Austin and Wolff, 1968; Krygier et al., 1982b). Although sinapine may have physiological functions during germination and maturation stages of canola and rapeseed (Bouchereau et al., 1992; Shahidi and Naczk, 1995), sinapine and the related phenolics are responsible for the tainting of brown-shelled eggs (Hobson et al., 1977; Goh et al., 1979; Shahidi and Naczk, 1992) when the meal was used as a poultry feed and possibly also for several physical and nutritional defects of the meal, such as color darkening (Keshavarz et al., 1977; Sosulski, 1979; Youngs, 1991). The presence of sinapine and the related phenolics such as sinapic acid, the hydrolyzed product of sinapine, has been a concern for oilseed breeders and processors (Blair and Reichert, 1984; Solsuski and Dabrouski, 1984). Rapid and sensitive methods for determining sinapine and related phenolics will facilitate canola/rapeseed breeding and processing. Thin layer chromatography (Ribereau-Gayon, 1972), which has been extensively used to separate phenolics, is difficult to quantitate. Colorimetric methods (Swain and Hillis, 1959; Austin and Wolff, 1968), when used to determine individual phenolics, require specific reagents for color development. Furthermore, it is difficult to avoid the interference from other compounds in these systems. Some colorimetric methods and ultraviolet spectrophotometric methods require purification procedures (Wang et al., 1998; Ismail and Eskin, 1979). Gas chromatography (Krygier et al., 1982b; Hartley and Jones, 1975) requires the hydrolysis of

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phenolic esters and the derivatization of the resulting phenolic acids before determination. Therefore, it is an indirect method and not directly applicable to nonvolatile phenolic esters themselves. HPLC is a rapid and sensitive method for qualitative and quantitative analysis of plant phenolics (Andersen, 1983).

Several HLPC methods for phenolic determination have been developed. Clausen et al. (1983) used a reverse-phase Nucleosil 5 C8 column and a Nucleosil 5 C18 column with a 30-minute linear gradient composed of acetonitrile and a phosphate buffer to separate a group of 22 standard aromatic choline esters. Hagerman and Nicholson (1982) used a Lichrosorb C8 column for a total 45 of minutes with two isocratic elutions of solvents composed of methanol and a sodium acetate buffer to separate hydrolyzed phenolic acids from plant extracts. Separations were excellent in both cases. These two methods, however, were tested only with either anionic or cationic phenolics and neither has been tested with intact plant extracts. With an uBondapak C18 column, Lattanzio (1982) used a combination of isocratic and linear gradient elution to separate about 30 standard phenolic acids and flavonoids, and used two concave gradients to separate about 14 standard flavonoids in 50 minutes. However, only hydrolyzed phenolic acids of eggplant extracts were tested using the retention times of the simple phenolic acids. Bjerg et al. (1984) used a series of ion-exchange columns to separate the phenolics into neutral, anionic, and cationic groups prior to HPLC analysis. Bouchereau et al. (1991; 1992) used the same technique to separate phenolics into different charged groups and then determined each group separately with a Spherisorb ODS 2 column. Although the HPLC separation for the anionic and the cationic fractions only took

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about 25 and 30 minutes, respectively, long hours were involved in concentration and purification during sample preparation.

Most of the previous methods either involved complicated purification steps or were used only for the separation of standards and hydrolyzed phenolic acids, which would not be applicable to many instances where the intact phenolics are the interest of the analysis. New techniques with simpler sample preparation procedures are required for intact phenolic analysis, especially when a large number of samples is to be analyzed. Mailer et al.(1993) used a HPLC method to qualitatively determine the ethanol extracts from canola flours as a means of cultivar identification. However, no quantitative determinations were made. The present method took advantage of the fact that the retention time of the individual phenolics in a system could be properly adjusted with a change in elution solvent composition. Since sinapate anion and sinapine carry opposite charges and have very different hydrophobicities, they are difficult to separate with reasonable retention times in the same solvent system. It was demonstrated with this study that it was possible to separate the anionic and cationic phenolics in a single run with the same solvent system by adjusting the solvent composition. The current method separated the anionic and the cationic phenolics in either a 10-min or a 15-min run with no purification steps. Therefore, it greatly increased the analytical efficiency and reduced cost. In addition, analytical results for the two phenolics with opposite charges were obtained from the same chromatogram, thus making them directly comparable.

MATERIALS AND METHODS

A. Sources of Materials

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sinapine (3,5-dimethoxy-4-hydroxycinnamoyl choline) was isolated from *Sinapis alba* certified seed from Tilney Mustard crop as sinapine bisulfate according to the method outlined by Clandinin (1961). This sinapine bisulfate was used in place of sinapine as a standard for HPLC calibrations. Parkland canola seed, a variety of *B. campestris*, was provided by ConAgra Seeds (AB, Canada). Canola meal was provided by CanAmera Foods (Fort Saskatchewan, AB, Canada). Canola protein isolate was prepared according to a protein micellar mass (PMM) procedure outlined by Murray et al. (1978), using the canola meal as raw material. CM Sephadex C-25 was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Ecteola Cellulose, capacity 0.31 meq./gm, was purchased from Sigma Chemical Company (St. Louis, MO). Folin-Ciocalteu's reagent was also purchased from Sigma Chemical Company. Acetic acid and sodium hydroxide were verified ACS-grade and obtained from Fisher Scientific Co. (Nepean, ON, Canada). The methanol used for canola phenolic extraction and HPLC analysis was HPLC-grade. All other chemicals, unless stated otherwise, were verified ACS-grade and purchased from Fisher Scientific Co. Deionized water was used throughout the research.

B. Methanol extraction of canola flour and meal

The canola flour was prepared by grinding the canola seed in a grinder (Scientific Industrial Inc. Bohemia, NY) for 1 min, extracting the ground material with hexane for 16 h with a Sohxlet apparatus and re-grinding the defatted flour for 1 min in the grinder. Canola meal was ground for 1 min in the grinder. The water contents of the flour and the meal were determined to be 6.0 and 7.2%, respectively. Fifty mg canola flour or meal were weighed into a 20-mL capped test tube for the first three extraction conditions or a 250 distilling vessel connected with a distilling/condensing system for the last extraction condition. Extraction was conducted under four conditions as follows: (a) 100% methanol, 50 °C, 10 min; (b) 100% methanol, 75 °C, 20 min and (c) 70% methanol, 75 °C, 20 min, plus (d) 100% methanol refluxing for 20 min. Except for the reflux system, temperatures were maintained using a Haake C Water Circulator (Karlsruhe, Germany). After each extraction, the mixture was cooled to room temperature before the cap was unscrewed. The liquid layer was decanted and the volume measured to check for vapor loss. Under normal extraction conditions used in this study there was no detectable vapor loss. The solid layer was discarded. The liquid phase was filtered through a 0.45-μm filter with a 3-mL syringe. This filtered liquid was used for both the HPLC analysis and total phenolic determination using the Folin-Ciocalteu's reagent method. The concentration of the liquid phase was assumed to be the same throughout the mixture and the volume of the solvent originally added to the sample was used in calculations (Wang, et al., 1998)

C. HPLC analysis

Chromatographic equipment consisted of two Waters (Milford, MA) pumps (model 501 and 510) and an automated gradient controller model 680, a Shimadzu (Kyoto, Japan) SPD-6A ultraviolet (UV) spectrophotometric detector, and a Hewlett-Packard (Avondale, PA) model HP3396II integrator connected with a peak 96 HPLC software. A reverse-phase C18 column (Supelcosyl, 3-µm particle size, 33x4.6 mm i.d.; Supelco, Bellefonte, PA) was used. Component A was a 0.05 M acetate buffer prepared by a 1:100 dilution of a stock pH 4.7 sodium acetate buffer. The stock buffer was prepared by adjusting 5 M acetic acid to pH 4.7 with solid NaOH (Hagerman and Nicholson, 1982). Component A was filtered through a 0.45-µm filter. Component B was 100% methanol. The column was maintained at 37 °C and run at a constant flow rate of 1.4 mL/min.

Two gradients were selected from a series of gradients examined and their solvent profiles are shown in Fig.5.1. In gradient A, the initial solvent was 15% methanol (component B) and 85% component A. After a 3.5-min isocratic flow, a 1.5-min linear gradient was used to change the solvent composition to 45% methanol. Then a 5-min concave gradient altered the solvent composition from 45% to 100% methanol. This solvent was maintained for 2 min until another 2-min linear gradient returned the solvent to its original composition (15% methanol). Sinapic acid was eluted during the 3.5-min isocratic flow while sinapine eluted with the 5-min concave gradient. In gradient B, the initial solvent was 14% methanol (component B) and 86% component A. After a 5-min isocratic flow, a 10-min linear gradient was used to change the solvent composition from 14% methanol to 100% methanol. This

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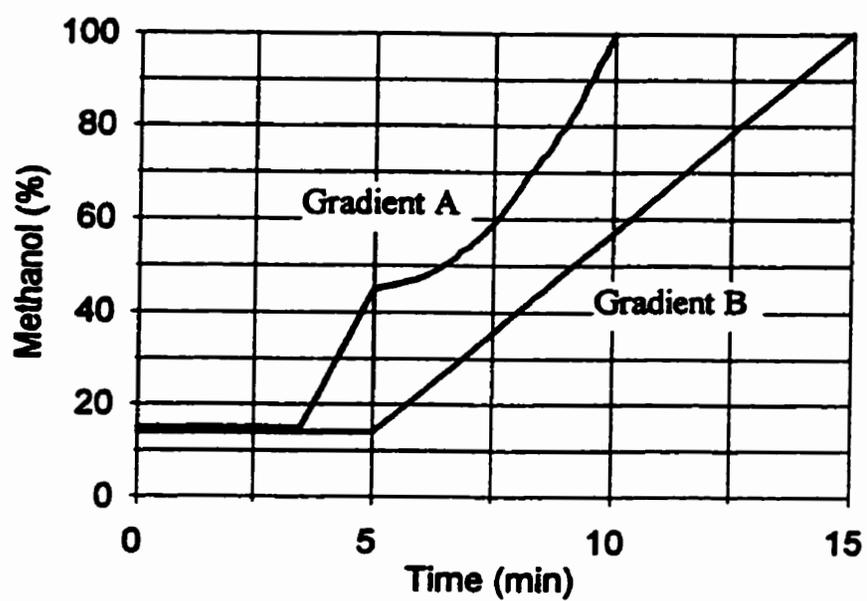
Fig.5.1. Elution solvent profiles for gradient A and B used in the HPLC method.

solvent was maintained for 2 min until another 2-min linear gradient returned the solvent to its original composition (14% methanol). Sinapic acid was separated during the isocratic flow while sinapine separated during the linear gradient elution. Other gradients tested included a combination of different concave and linear gradients with varying elution times but provided no improvement in separation of the phenolics.

D. Ion-exchange

About two grams of CM-Sephadex C25 resin were treated with 10 mL 1M HCl and then washed with water until the pH of the eluant was neutral. The treated resin was then packed by gravity into a 10-mL pipet to form a cation-exchange column (8x60 mm). About two grams of Ecteola Cellulose were treated with 10 mL 2 M acetic acid and washed and packed as described above to form an anion-exchange column (8x60 mm) (Bjerg et al., 1984).

Fifty mg of the ground canola flour were refluxed with 5 mL 100 % methanol for 20 min. After cooling to room temperature, the liquid layer was filtered through a 0.45 μm filter. The liquid phase was then concentrated to about 200 μL by evaporating under vacuum. This concentrate was first applied to the CM-Sephadex C-25 cation-exchange column and washed with 10 mL water. The effluent was placed on the Ecteola Cellulose anion-exchange column. Neutral phenolic derivatives were collected in the effluent from the anion-exchange column. Aromatic choline esters were eluted from the cation-exchange column, first with 10 mL of a mixture of 2 M acetic acid/methanol (1/1, v/v), and then with 10 mL 100% methanol. Acidic phenolic acids were eluted from the anion-exchange column using 1 M pyridine (Fig.5.2). All

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the effluents were analyzed by HPLC.

A. Cation-column

CM-Sephadex C-25

-OCH₂COO⁻

B. Anion-column

Ecteola-cellulose

-(OCH₂CH₂)₃ NH

Eluted withH₂O
Neutral fraction

A.

B.

Eluted with
(1) 2N acetic acid/methanol
(1/1, v/v), (2) 100% methanol
Cationic fraction

Eluted with 1M pyridine
Anionic fraction
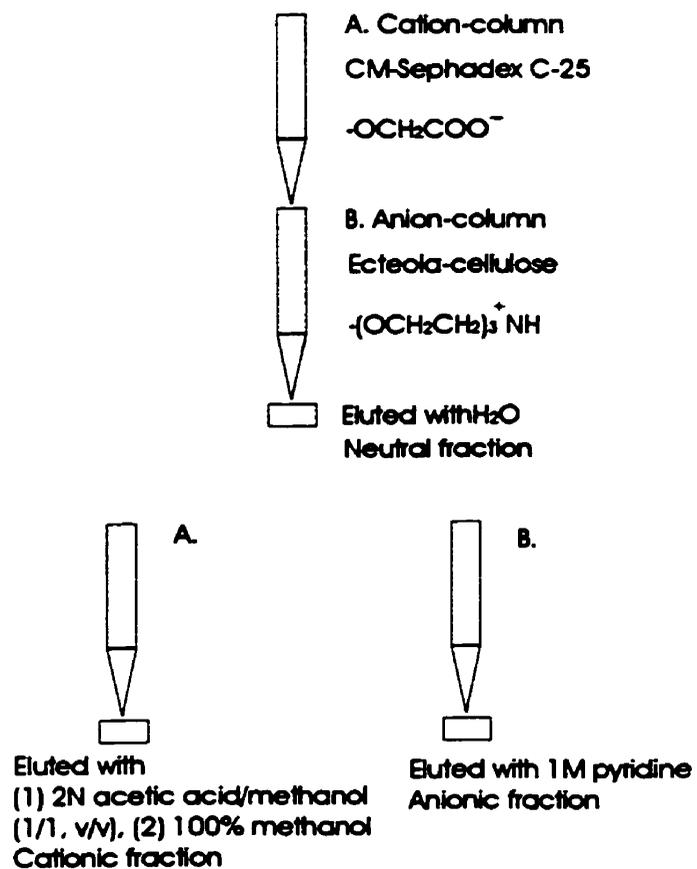
Fig.5.2. Elution principles of two different types of ion-exchange columns.

E. Standard calibration

Stock solutions (200 μg/mL) for sinapine bisulfate and sinapic acid were prepared by accurately weighing 1 mg of each substance into 5 mL methanol. Dilution of the above stock solutions gave two sets of standard solutions of 200, 100, 50 and 25 μg/mL for sinapine bisulfate and sinapic acid, respectively. During the calibration, a 3-μL mixture of sinapine bisulfate/sinapic acid solution (2 μL sinapine bisulfate and 1 μL sinapic acid) was injected into the HPLC column using a 25-μL sample loop. Two calibration curves were obtained for sinapine bisulfate and sinapic acid, respectively, by plotting concentrations versus peak areas. Regression equations were obtained from the calibration curves for the two compounds.

F. Calculation of sinapine and sinapic acid content

Sinapine and sinapic acid contents of the flour and meal were calculated using the following equations:

$$sinapine\,bisulfate(mg/g)=(a+bA)\frac{Vc}{Vs}\frac{Vt}{W}$$

$$sinapic\,acid(mg/g)=(a+bA)\frac{Vc}{Vs}\frac{Vt}{W}$$

$$sinapine\ (mg/g) = sinapine\ bisulfate\ *(310.4/407.4)$$

where a and b = y-intercept and slope of the standard curves for sinapine bisulfate or sinapic

acid, A = peak area, Vc = injection volume for calibration (μL), Vs = injection volume for sample (μL), Vt = volume of solvent added to sample (mL), W = weight of the flour or meal (mg), 310.4 = molecular weight of sinapine, 407.4 = molecular weight of sinapine bisulfate.

G. Total phenolic content

The total phenolic content was determined by Folin-Ciocalteu's reagent method according to Swain and Hillis (1959) and Schanderl (1970). Total phenolic content was expressed as tannic acid equivalents. When estimating the total phenolic content using the total peak area from HPLC, total phenolic content was expressed as sinapine bisulfate equivalents. The total peak area from HPLC included sinapic acid, sinapine and all other unknown peaks. Total phenolic contents estimated by this method were found to be very close to that determined by the Folin-Ciocalteu's reagent method.

H. UV spectra and extinction coefficients of standard sinapine bisulfate and sinapic acid

UV spectra and extinction coefficients of standard sinapine bisulfate and sinapic acid were determined with a Hewlett-Packard 8452 diode array spectrophotometer with MS-DOS UV-VIS software. Extinction coefficients were calculated according to the equation

$$\varepsilon = A/cl$$

where ε = extinction coefficient ($Lcm^{-1}mol^{-1}$), A = absorbance, c = concentration (mol/L) and l = cell length (cm).

RESULTS AND DISCUSSION

A. Selection of extraction conditions

Determination of optimal extraction conditions for the determination of sinapine and sinapic acid is a necessary prerequisite for their determination by HPLC. Bouchereau et al. (1992) extracted rapeseed phenolics by boiling the flour in 100% methanol. The phenolics in the extract were then separated by ion-exchange chromatography and determined by HPLC. On the other hand, 70% aqueous methanol was used for an extraction at 75 °C for 20 min during the determination of sinapine by UV light spectrophotometric method in conjunction with an ion-exchange separation (Wang et al., 1998). Naczk et al. (1992) found 70% aqueous methanol extracted twice as much total phenolics from rapeseed meal as pure methanol. Since the extractions were conducted at a solvent to meal ratio of 10:1, the extraction efficiencies of these solvents at a higher solvent to meal ratio (100:1 in this research) were still unknown. In addition, aqueous methanol extracts traces of proteinaceous material that would require removal before determination by HPLC. Due to the uncertainty of the efficiency of these extraction methods, they needed to be reviewed. In particular, the condition of aqueous methanol or pure methanol is a major point that needed to be clarified.

The effect of extraction conditions on the amount of sinapine, sinapic acid and total phenolics measured is given in Table 5.1. The amount of sinapine, sinapine bisulfate, sinapic acid and the total phenolics determined using the extraction condition of refluxing with 100%

methanol for 20 min were not significantly different from these determined using the extraction condition of 70% methanol at 75 °C for 20 min, but were significantly higher than those determined using the other two conditions at 50 °C and 75 °C with 100% methanol.

Table 5.1. Effect of extraction conditions on the contents of sinapine, sinapic acid and total phenolics of canola flour (dry basis) [a]

| mg/g material | 100% methanol | | | 70% methanol |
	50 °C, 10 min	75 °C, 20 min	Reflux, 20 min	75 °C 20 min
Sinapine	10.85 ± 0.26^B	10.65 ± 0.07^B	12.09 ± 0.72^A	11.74 ± 0.99^{AB}
Sinapine bisulfate	14.24 ± 0.34^B	13.99 ± 0.11^B	15.86 ± 1.04^A	15.40 ± 1.31^{AB}
Sinapic acid	0.36 ± 0.05^B	0.34 ± 0.05^B	0.49 ± 0.04^A	0.40 ± 0.03^{AB}
Total phenolics (HPLC)	28.56 ± 2.31^B	26.78 ± 2.07^B	32.16 ± 1.18^A	34.32 ± 1.86^A
Total phenolics (Folin-Ciocalteu's reagent)	28.84 ± 2.78^B	27.84 ± 2.09^B	35.19 ± 3.1^A	34.63 ± 2.84^A

[a] Mean of three replicates$\pm$SD. Row values with the same letters were not significantly different ($p \leq 0.05$).

Moreover, 70 % aqueous methanol extract contained some proteinaceous material. Although the filtered aqueous methanol extract was directly and successfully applied to the HPLC column in this research, it was suspected that the life time of the column would be

shortened if a large number of samples was analyzed. On the other hand, 100% methanol refluxing for 20 min appeared to not extract the proteinaceous material. It was concluded that 100% methanol, refluxing for 20 min, was the best extraction condition and this condition was, therefore, used as a routine method for the extraction of the phenolics. The other two methods gave incomplete extraction of phenolics.

The total phenolic contents estimated by HPLC coincided well with the total phenolic content determined by the Folin-Ciocalteu's reagent method.

B. Elution conditions

HPLC resolution is directly proportional to the difference between retention times of adjacent peaks over the sum of peak widths at the base of each peak (Macrae, 1982). Retention time is a thermodynamically controlled factor while peak width is a factor controlled by the kinetics of the solute. The goal of this study was to change the retention time by changing thermodynamic factors, (i.e., solvent composition) in order to obtain retention times different enough for good resolution, yet in a short chromatographic period. It was also observed that a change in solvent composition affected the peak width of sinapine.

Elution conditions were selected based on trial and error methods within the limits of the chromatographic system. The conditions tested included several isocratic, linear and nonlinear gradients, and the combinations of either two or all three of these gradients. Two elution conditions, gradient A and B (Fig.5.1), were selected.

HPLC chromatograms of different samples under these two elution conditions are

shown in Fig.5.3a-b. The three chromatograms (A, B and C) in each figure represent chromatograms of three samples from different extraction conditions, including 70% aqueous methanol at 75 °C for 20 min, 100% methanol refluxing for 20 min and 100% methanol at 50 °C for 10 min. The chromatogram of the sample extracted with 100% methanol at 75 °C for 20 min resembled that of the sample extracted with 100% methanol at 50 °C for 10 min and is not shown.

The number of peaks eluted by the two gradients was generally the same (Fig.5.3a and b). The combination of an isocratic and a linear gradient, gradient B (Fig.5.3b), gave better resolution than gradient A (Fig.5.3a), which is a combination of an isocratic, a linear, and a concave gradient. However, the time required for gradient B (15 min) was longer than that for gradient A (10 min). Since resolution of sinapine and sinapic acid from other compounds was good enough in both cases and gradient A required a shorter time, gradient A was used as a routine technique for the determination of sinapine and sinapic acid content. Other gradients tested in this study did not provide any better resolution or any better peak shape and they are not presented.

Although the number of peaks eluted with either gradient was also the same for all three samples extracted at different conditions (Fig.5.3a and b), there were differences in peak heights and areas for different samples. The sample extracted by refluxing with 100% methanol had the largest peak area for sinapine (peak 8) and sinapic acid (peak 5), whereas samples extracted with 70% aqueous methanol had the largest areas for peak 1.

With this method, both sinapine and sinapic acid are shown in the same
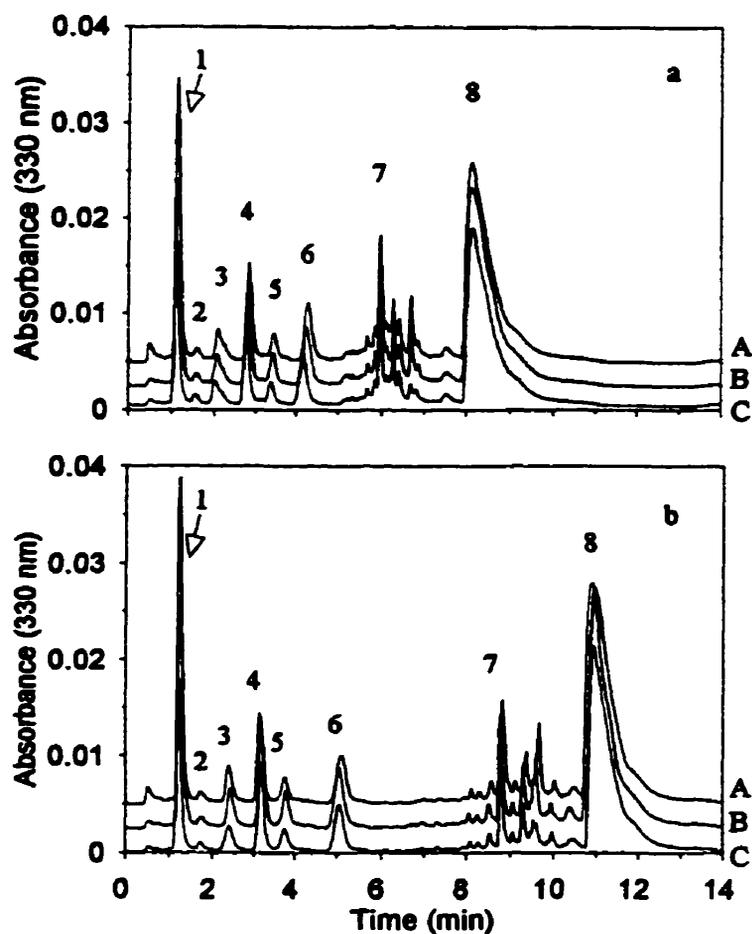
Fig.5.3a-b. HPLC chromatograms of different samples (A, B and C) using gradient A (a) and gradient B (b). Sample (A), extracted with 70% aqueous methanol at 75 °C for 20 min; (B), extracted with 100 % methanol refluxing for 20 min; and (C), extracted with 100% methanol at 50 °C for 10 min. Peak identifications: 5. sinapic acid, 8. sinapine; other peaks were unidentified.

chromatograms, and as a result, their levels are comparable, since the ratio of their extinction coefficients at 330 nm has been determined to be 1:1.16. In addition to being fast and simple, this direct comparison is another advantage of the current method over the conventional methods.

C. Ion-exchange

To verify the peak identities of sinapine and sinapic acid and to classify the other peaks into differently charged groups for possible peak identifications, the same extracts were separated using ion-exchange columns and analyzed using gradient A by HPLC. The extract was separated into neutral, anionic and cationic fractions before analysis by HPLC. The chromatograms of these fractions are shown in Fig.5.4a-d. Peaks 1 and 3 (Fig.5.4a) from the neutral fraction, which passed through anionic and cationic columns with water as the eluant, were possibly neutral compounds. According to Bouchereau et al. (1992), neutral compounds such as sinapoylglucose, 1,2 disinapoylglucose and sinapoylmalate have been found in methanol extracts of rapeseed flour. Peaks 2 and 5 (Fig.5.4b) come from the anionic fraction, which represent materials removed from the anionic exchange column eluted with 1 M pyridine. Peak 5 was identified as sinapic acid. Peak 2 could also be an anionic phenolic but was not identified, as was the case for the other unnumbered peaks in the chromatogram. Peaks 4, 6 and 7 (Fig.5.4c), from the first cationic fraction eluted from the cationic exchange column with 2 M acetic acid/methanol solvent, were possibly cationic compounds. Cationic compounds such as 4-hydroxybenzoylcholine (Clausen et al., 1982) and sinapine-O-β-D-

Fig.5.4a-d. HPLC chromatograms of fractions separated with ion-exchange
columns. (a) neutral fraction eluted with water, (b) anionic fraction eluted
with 1 M pyridine, (c)1st cationic fraction eluted with 2 M acetic
acid/methanol (1/1, v/v), (d) 2nd cationic fraction eluted with methanol.
Peak identifications: 5, sinapic acid; 8, sinapine; other peaks were
unidentified.

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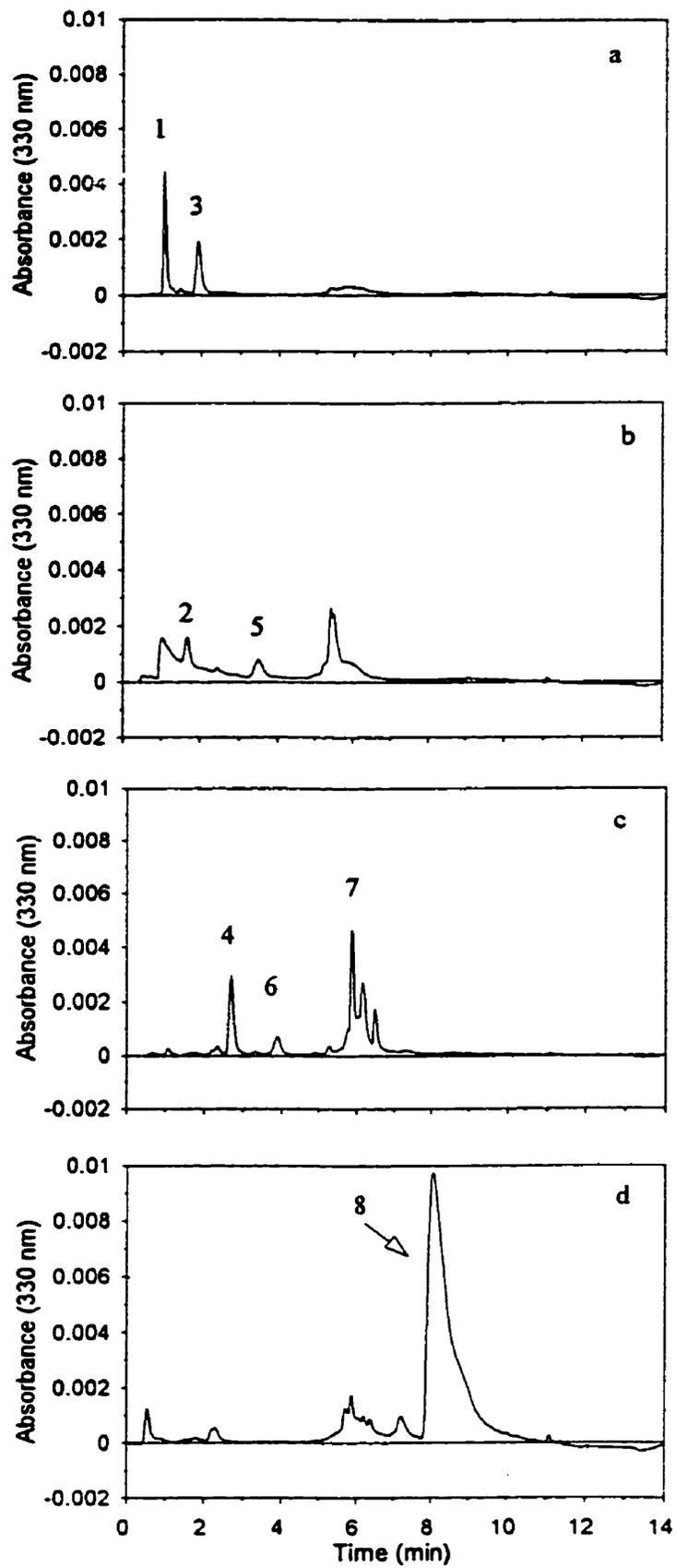
glucopyranoside (Larsen et al., 1983; Bouchereau et al., 1992) have been found to be present in seeds of some glucosinolate-containing plants. Peak 8 (Fig.5.4d) from the second cationic fraction, eluted with 100% methanol, was identified as sinapine. With the ion-exchange technique, it was verified that sinapic acid was in the anionic fraction while sinapine in the cationic fraction. Other unknown peaks were grouped into different charged groups. These peaks were, however, unidentified at this point. Attempts were made to identify these peaks by spiking the methanol extract of canola flour with standards of ferulic acid and p-coumaric acid (Appendix 5.1; 5.2), but none of the unknown peaks could be identified as either of these two compounds. Similarly, none of the unknown peaks could be identified as p-hydroxybenzoic acid.

According to Fig.5.4a-d, it can be seen that there was no evidence of other compounds that eluted at the same time as either sinapine or sinapic acid. This indicated that there was no risk of the contamination from other compounds, which could result in an overestimation based on peak area.

D. Standard chromatograms

HPLC chromatograms for the standard sinapine bisulfate and sinapic acid analyzed using gradient A are shown in Fig.5.5. The four curves show chromatograms resulting from using the same injection volume (2 μL sinapine bisulfate and 1 μL sinapic acid) but different concentrations. The shapes of the peaks were symmetric with sinapic acid, but skewed to the left for sinapine bisulfate. Clausen (1983) obtained a better peak shape with a Nucleosil C5
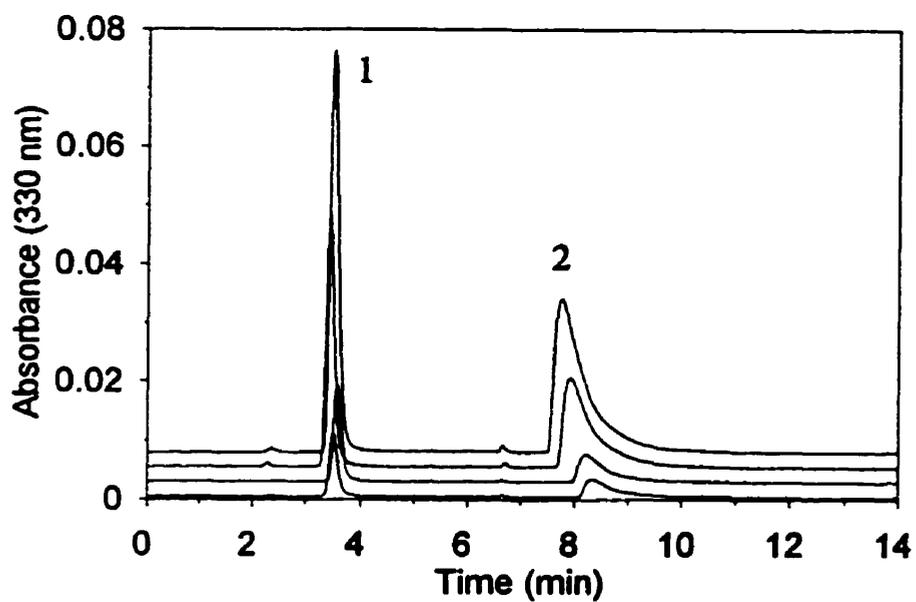
Fig.5.5. HPLC chromatograms of a mixture of standard sinapine and sinapic acid. From top to bottom 200 µg/mL, 100ug/mL, 50 µg/mL and 25 µg/mL sinapine and sinapic acid each in a mixture of 3 µL injection volume (2 µL sinapine and 1 µL sinapic acid).

column than a Nucleosil C18 column when separating aromatic choline esters. The present method, using a reverse phase C18 column, gave good results despite the asymmetric shape of the sinapine bisulfate peak.

The retention times from the chromatographic studies are shown in Table 5.2. The analysis of variance showed that the retention times for sinapine bisulfate were significantly different ($p \leq 0.05$). Retention time increased as concentration decreased. On the other hand, retention times for sinapic acid were not significantly different ($p \leq 0.05$).

E. Calibration curves

Peak areas in relation to concentrations of standard sinapine bisulfate and sinapic acid (calibration curves) are shown in Fig.5.6a-b. Linear regression analysis showed that for both sinapine bisulfate and sinapic acid there was a linear relationship between peak area and concentration. Therefore, the determination of concentration according to peak area is valid. Regression equations for the two compounds are as follows:

sinapine bisulfate: $Y \text{ (spn)} = 0.2022 + 4 \times 10^{-5} X$

sinapic acid: $Y \text{ (spc)} = 0.9597 + 4.8 \times 10^{-5} X$

where: $Y \text{ (spn)}$ = sinapine bisulfate concentration ($\mu g/mL$), $Y \text{ (spc)}$ = sinapic acid concentration ($\mu g/mL$), and X = peak area (arbitrate).
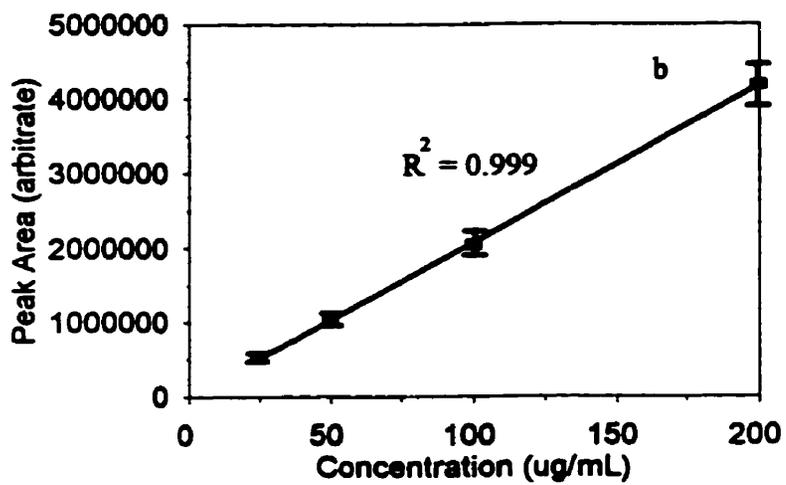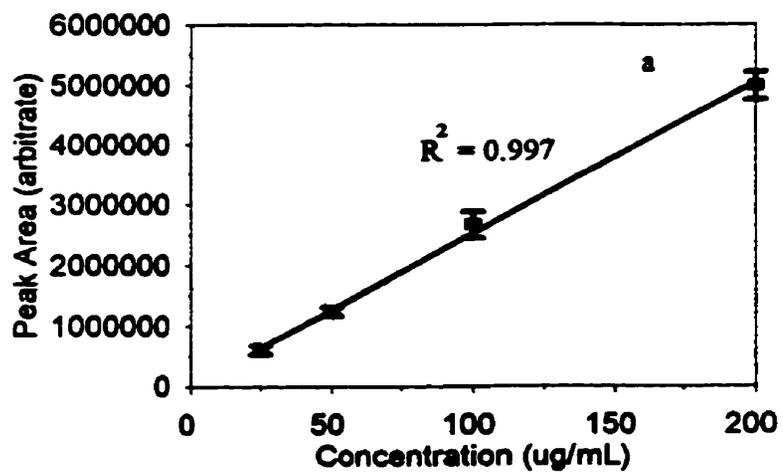
Fig.5.6a-b. Standard calibration curves for (a) sinapine and (b) sinapic acid.

Table 5.2. Retention times in relation to concentrations of standard sinapine bisulfate and sinapic acid (2 μL of sinapine bisulfate and 1 μL sinapic acid solutions) [a]

	Retention time (min)	
Concentration (μg/mL)	Sinapine bisulfate [b]	Sinapic Acid [b]
200	7.844±0.108 [A]	3.607±0.081 [A]
100	8.000±0.111 [B]	3.532±0.195 [A]
50	8.254±0.049 [C]	3.605±0.169 [A]
25	8.414±0.088 [D]	3.533±0.122 [A]

[a] Mean of six replicates±SD.

[b] Column values with the same letters were not significantly different ($p \leq 0.05$).

F. UV spectra and extinction coefficients of sinapine bisulfate and sinapic acid at detector wavelength

UV spectra of sinapine bisulfate and sinapic acid are given in Fig.5.7. The maximum absorbance wavelengths for sinapine bisulfate and sinapic acid were 332 and 322 nm, respectively. Since sinapine is the major phenolic component of canola/rapeseed, the detector wavelength was set close to the sinapine maximum (330 nm). In addition, this wavelength has been used in previous determinations of sinapine (Wang et al, 1998) and sinapic acid (Hagerman and Nicholson, 1982). To compare sinapine and sinapic acid on the same chromatogram, their extinction coefficients at the maximum absorbance wavelengths and at
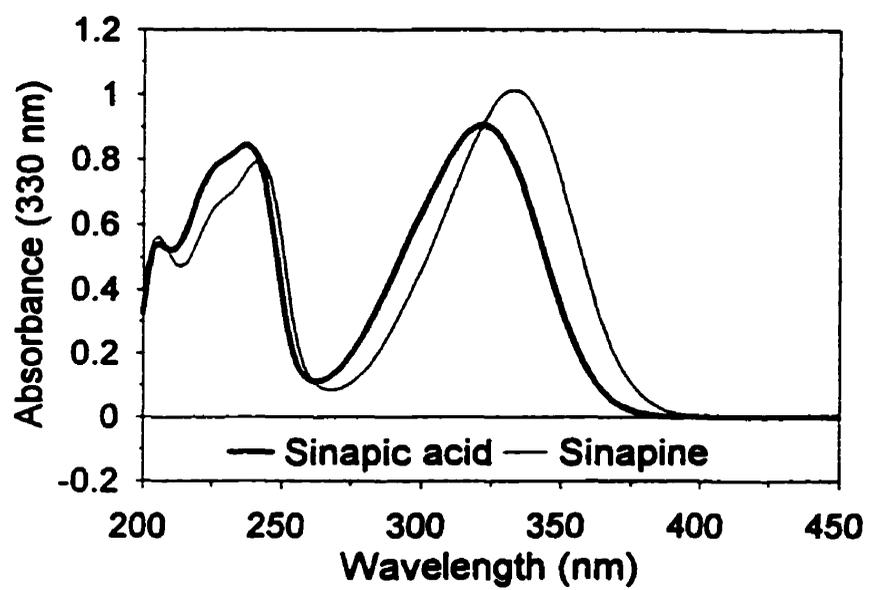
Fig.5.7. UV spectra of sinapine and sinapic acid.

the detector wavelength were determined. These values are listed in Table 5.3. The extinction coefficients for sinapine bisulfate and sinapic acid at 330 nm were 2500±53 and 2147±61 cm⁻¹mol⁻¹, respectively. Therefore, the absorbance of sinapine was 1.16 times higher than that of sinapic acid (mole concentration basis). Similarly, the same peak areas for sinapine and sinapic acid on the chromatogram should indicate a mole ratio of 0.86 to 1.

Table 5.3. Maximum absorbance wavelengths (λ_{max}) and extinction coefficients of sinapine bisulfate and sinapic acid at λ_{max} and at detector wavelength (λ_{330}) in methanol ^{a}

	λ_{max} (nm)	ε_{max} (cm⁻¹mol⁻¹)	ε_{330} (cm⁻¹mol⁻¹)
Sinapine bisulfate	332	2518±53	2500±53
Sinapic acid	322	2300±60	2147±60

^{a} Mean of three replicates±SD.

G. Determination of the phenolic contents of the methanol extracts from canola whole seed, industrial meal and protein isolate

The sinapine and sinapic acid contents of canola flour, canola industrial meal and a protein isolate (PMM) were determined by HPLC using gradient A. The results are given in Table 5.4. The sinapine content was 12.03, 11.38 and 0.74 mg/g for canola flour, industrial meal and the protein isolate, respectively. Note that the results of the flour and meal were not directly comparable since the sources of the materials were different. PMM was isolated from

the same meal used in this research, therefore, after isolation, the PMM contained less than one tenth of its original sinapine. The variations in retention times were consistent with those observed for the standards. Also note that since standard sinapine bisulfate was used in place of sinapine, the results must be converted from sinapine bisulfate to sinapine. The values of sinapine bisulfate equivalents were also included in parentheses in Table 5.4. Retention times for sinapic acid were approximately 3.4 min for all the three materials, similar to that seen for the standard.

According to the literature (Shahidi and Naczk, 1992), a higher content of sinapine was found in *Brassica napus* rapeseed cultivars (16.5 - 22.6 mg/g) than in *Brassica campestris* cultivars (12.2 - 15.4 mg/g). In other studies (Blair and Reichert, 1984), 26.7 and 28.5 mg/g of sinapine were found in defatted rapeseed and canola cotyledons. In a colorimetric method using titanium tetrachloride for the determination of sinapine in rapeseed, the content of sinapine was 10.4 mg/g for rapeseed and 1.1 to 1.8 mg/g for rapeseed protein concentrate (Ismail and Eskin, 1979).

The total content of free phenolic acids was only 0.06 mg/g of flour in the Indian cultivar, Yellow Sarson. Canadian cultivars, Candle and Tower, contained over 10 times this level, primarily due to the high levels of sinapic acid (Krygier et al., 1982b).

Therefore, the sinapine and sinapic acid contents determined by the current method are in the same range as values previously reported in literature.

Table 5.4. Retention times and sinapine and sinapic acid contents of canola flour, industrial meal and canola protein isolate (PMM) (dry basis) [a]

| Source | Sinapine | | |
	Retention Time (min)	Content (mg/g)	(Sinapine bisulfate) (mg/g)
Flour	7.964±0.048	12.03±0.63	(15.79±0.82)
Meal	8.007±0.040	11.38±1.35	(14.95±1.78)
PMM	8.520±0.208	0.74±0.22	(0.97±0.29)

| | Sinapic acid | |
Source	Retention time (min)	Content (mg/g)
Flour	3.487±0.052	0.39±0.05
Meal	3.439±0.036	0.24±0.06
PMM	3.384±0.112	0.20±0.06

[a] Mean of five replicates±SD.

In conclusion, the current method provides a means of separating sinapine and sinapic acid from other phenolics using a 10-min or a 15-min gradient on a reverse phase C 18 column. Ion-exchange techniques in conjunction with HPLC analysis verified that sinapine and sinapic acid were in cationic and anionic fractions, respectively, and that their peaks were

not contaminated. This method simplified the sample preparation and chromatographic

procedures in comparison with previous methods. In addition, the peaks of sinapine and

sinapic acid could be compared on the same chromatograms.

CHAPTER VI. THE EFFECT OF PROCESSING CONDITIONS AND THE PRESENCE OF SINAPINE BISULFATE AND SINAPIC ACID ON THE COLOR OF CANOLA PROTEIN ISOLATE

CHAPTER VI. COLOR OF CANOLA FLOUR AND ISOLATE

ABSTRACT

The influence of phenolic compounds and processing conditions on the color of canola protein isolates and the effect of autoclaving on dehulled and whole seed canola flour were investigated. Protein isolates prepared using three different methods, aqueous NaCl extraction with acidic precipitation (AAP), aqueous NaCl extraction with precipitation by dilution (PMM) and basic extraction with acidic precipitation (BAP), were compared for their colors and phenolic profiles. In addition, sinapine bisulfate and sinapic acid were added to a protein isolate containing minimum phenolic levels, and color and phenolics evaluated following autoclaving or basic extraction simulated conditions. Autoclaving resulted in a significant darkening of both flours. The addition of sinapine bisulfate during autoclaving increased the darkening slightly while the addition of sinapic acid increased the intensity of yellow component. The isolate prepared with the BAP procedure gave the darkest color with the highest level of sinapine. The addition of sinapine bisulfate or sinapic acid prior to the exposure to alkali, however, did not have a negative impact on the color of the canola protein isolate. The darkening during alkaline extraction, therefore, was probably not due to sinapine or sinapic acid or compounds produced from these phenolics under basic conditions.

Key Words: alkali, autoclaving, basic condition, canola protein isolate, color, phenolics, processing, sinapic acid, sinapine.

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CHAPTER VI. COLOR OF CANOLA FLOUR AND ISOLATE

INTRODUCTION

To increase the uses of canola protein, the causes of a dark color for the meal and the canola protein isolated during processing need to be investigated. The effect of some processing conditions on the color of canola protein isolate has been investigated by several researchers (Youngs, 1991; Keshavarz et al., 1977). During seed extraction to remove oil, the severe moist-heat treatment in the cooker and the desolventizer, darkened the meal color and denatured the protein (Youngs, 1991). When several processing conditions were compared during canola protein isolation, higher pH conditions produced a darker isolate than did lower pH conditions (Keshavarz et al., 1977). Even when a protein micellar mass (PMM) procedure was employed, where the extraction and precipitation were conducted at an acidic condition (around pH 6), the color of the product was often not as light as desired for food uses. Chemical modifications such as acetylation and succinylation appeared to improve the color of the PMM (Gruener and Ismond, 1997a) in addition to their effects on functional and physiochemical properties of the PMM (Gruener and Ismond, 1997b). Many previous reports frequently attributed the dark color to the presence of phenolics (Shahidi and Nazck, 1992; 1995). However, there appeared to be no report on whether sinapine and sinapic acid, the major phenolics in canola, were directly responsible for the dark color of these products. Neither had there been any interpretation concerning how the color compounds were developed.

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In a series of investigations looking at the structural changes and coloration of canola phenolics, the structural changes in sinapic acid during autoclaving were found to produce several colored compounds that caused a coloration for the system (Cai et al., 1999a). However, neither structural changes nor color development were found in sinapine bisulfate solutions under the same autoclaving conditions. In view of the fact that the content of sinapic acid in the meal is only around 0.4 mg/g while that of sinapine is as high as 12.0 mg/g, it is of interest to determine the contribution of these phenolics to the color of the protein isolates. An investigation on the phenolics in determining the color of the meal and protein systems may provide further insights into the mechanism of color development.

Apart from the autoclaving experiments, controversies were also noted for the role of sinapine and sinapic acid on the color of protein isolated during alkaline isolation. A dark isolate was produced at alkaline conditions (pH 10) within one hour of extraction, while the 2,6-dimethoxyl-p-benzoquinone, a colored compound from sinapic acid during air oxidation at pH 10, was not detected until 169 hours in phosphate-boric acid buffers or 72 hours in ammonium bicarbonate buffers (Cai et al., 1999b). Structural changes and color intensity in sinapine bisulfate solution were noticeable after 24 hours (Chapter IV). These results seemed to indicate that the development of colored compounds from sinapic acid or sinapine bisulfate required a time much longer than that needed for protein isolation. This may further indicate that the dark colored meal and protein isolate were not caused by the structural changes of sinapine and sinapic acid. There is a need to determine the effect of processing conditions and the presence of sinapine and sinapic acid on the color of meal and protein isolate.

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In the present research, the protein isolates from several isolation procedures were compared for color, phenolic and protein contents to evaluate the effect of processing conditions on the color of canola protein. The effects of autoclaving on the color of canola flour were investigated in both whole seed flour and dehulled canola flour. The effect of sinapine bisulfate and sinapic acid on the color of protein isolate was determined by conducting several experiments under autoclaving and alkaline extraction conditions using a mixture of protein isolate and phenolics.

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MATERIALS AND METHODS

A. Sources of materials

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sinapine (3,5-dimethoxy-4-hydroxycinnamoyl choline) was isolated from *Sinapis alba* certified seed from Tilney Mustard crop as sinapine bisulfate according to the method outlined by Clandinin (1961). Parkland canola seed, a variety of *B. campestris*, was provided by ConAgra Seeds (AB, Canada). Canola meal was provided by CanAmera Foods (Fort Saskatchewan, AB, Canada). Folin-Ciocalteu's phenol reagent was purchased from Sigma Chemical Company (St. Louis, MO). Acetic acid and sodium hydroxide were verified ACS-grade and obtained from Fisher Scientific Co. (Nepean, ON, Canada). The methanol used for canola phenolic extraction and HPLC analysis was HPLC-grade. All other chemicals, unless stated otherwise, were verified ACS-grade and purchased from Fisher Scientific Co. (Nepean, ON, Canada). Distilled water was used for protein isolation.

B. Protein isolation

Protein isolates were prepared from canola meal following three methods described below:

a. Aqueous NaCl extraction with acidic precipitation (AAP method, Owen et al., 1972)

Ten grams of canola meal were dispersed into 100 mL of 0.5 M NaCl solution. The

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mixture was stirred mechanically for one hour at room temperature (22 °C). The slurry was centrifuged at 3000xg for 10 min at 5 °C. The supernatant was collected and the solid discarded.

The pH of the supernatant was adjusted to 2.5 with 6 M HCl to precipitate the proteins, which were allowed to settle. The supernatant fluids were then decanted and discarded. To remove impurities, the protein precipitate was washed three times using 150 mL of fresh water for each wash. The final precipitate was centrifuged at 3000xg for 10 min at 5 °C. After the liquid phase was removed, the solid phase was freeze-dried to yield the protein isolate.

b. Aqueous NaCl extraction with precipitation by dilution (PMM method) (Murray et al., 1978; Ismond and Welsh, 1992)

Canola protein isolate was prepared according to a protein micellar mass (PMM) procedure outlined by Murray et al. (1978) and modified by Ismond and Welsh (1992). Meal samples weighing 3.5 kg were stirred with 35 L of 0.5 M NaCl solution for four hours to extract protein in a large kettle equipped with an overhead lighting mixer. The extracted protein solution was centrifuged to remove the solid residue using a Sorvall Refrigerated Centrifuge (model RC-3, Ivan Sorvall Inc. Norwalk. CT., USA) at 5 °C and 3,000xg for 12 min. The solution was then concentrated with an Amicon Hollow Fiber cartridge ultrafiltration unit (H10P30-20, Amicon Corporation, Danvers, MA, USA) with a 30,000 molecular weight cut-off, operating with an inlet pressure of less than 140 kPa (20 psi) and

a back-pressure of 50 kPa (0.5 bar). The volume of the solution was reduced approximately 16 fold. The concentrate was then diluted by 15 times its volume with cold tap water to produce the protein micellar mass, which was then collected by centrifugation and freeze-dried to yield the protein isolate.

c. Basic extraction with acidic precipitation (BAP method) (Gillberg and Tornell, 1976a; 1976b; Keshavarz et al., 1977)

Ten grams of canola meal were dispersed into 100 mL distilled water. The pH value of the slurry was adjusted to 8.5 or 10 with 1 N NaOH. The mixture was stirred mechanically for one hour at room temperature (22 °C). The pH value was readjusted several times to ensure that it remained at the required value. The final slurry was centrifuged at 3000xg for 10 min at 5 °C. The supernatant was collected and the solid discarded.

The supernatant was adjusted to pH 4 with 1 M HCl to precipitate the proteins, which were allowed to settle. The liquid phase was then decanted and discarded. To remove impurities, the protein precipitate was washed three times using 150 mL of fresh water for each wash. After the liquid phase was removed, the final precipitate was centrifuged at 3000xg for 10 min at 5 °C. The solid phase was freeze-dried to yield the protein isolate.

C. Preparation of canola flour for assessing the effect of autoclaving on the color of the flour

Both dehulled and whole seed flours were prepared from whole canola seed.

Dehulling. About 500 g Parkland canola seed were heated at 70 °C for 20 min in a constant temperature cabinet (Stabil Therm, Blue M Electric Company). The seeds were then cooled for about 10 min before applying a gentle force by rolling the seeds with a beaker to crack the seeds. Further separation of hulls and kernels was done by hand. Both clean kernels and hulls were kept in clean bottles for further experiments.

Grinding and extraction. Both dehulled and whole seed flours were prepared by grinding the whole seed or dehulled kernel in a grinder (Scientific Industrial Inc. Bohemia, NY) for 1 min, extracting the ground material with hexane for 16 h in a Sohxlet apparatus and re-grinding the defatted flours for 1 min.

Autoclaving. Autoclaving was conducted using an AMSCO Eagle 3000 Series Sterilizer (American Sterilizer Company, Ho PA). Time, temperature, and pressure were controlled automatically. A gravity model was used for the autoclaving of the samples, where no liquid water was added during autoclaving. Temperature and pressure were routinely set to 121 °C (250 F) and 0.1 MPa (15 Psi).

D. Determination of protein content

The protein content was determined by a Kjeldahl nitrogen method using a Kjeltec Auto 1030 Analyzer (Tecator AB, HOGANAS, Sweden).

E. Determination of the color of protein isolates

The color of the protein isolates was determined with a HunterLab Color/Difference

Meter, model D25-2 (Hunter Associates Laboratory, Inc. Fairfax, VA. USA).

F. Determination of soluble and insoluble-bound phenolics

Krygier et al. (1982) grouped the canola phenolics into three categories, i.e., free phenolics (e.g., sinapic acid), esterified phenolics (e.g., sinapine) and bound phenolics (e.g., tannin). In the current study, the terms soluble and insoluble-bound phenolics as defined by Hahn et al. (1983) are used. Soluble phenolics are therefore defined as phenolics soluble in methanol (e.g., sinapic acid and sinapine), and the insoluble-bound phenolics are defined as phenolics liberated after alkaline hydrolysis (e.g., sinapic acid liberated from cell wall).

Soluble sinapine and sinapic acid were determined using a rapid HPLC method (Chapter V). The determination was calibrated using sinapine bisulfate and sinapic acid standards. Total soluble phenolic and insoluble-bound phenolic contents were also determined by both the rapid HPLC method (expressed as sinapine bisulfate equivalent) and by Folin-Ciocalteu's reagent method (Velioglu et al., 1998; Pearson, 1971; Swain and Hillis, 1959) (expressed as tannic acid equivalent). Unless stated otherwise, 100% methanol was used as solvent for the phenolic extraction from flour and meal.

Samples for assessing the insoluble-bound phenolic content were prepared according to the procedure outlined by Krygier et al. (1982b). The canola meal was washed 3 times with 70% aqueous methanol and another 3 times with 70% aqueous ethanol (1/100, w/v) at room temperature. Fifty mg of this washed canola meal were then hydrolyzed with 5 mL (1/100, w/v) 4 N NaOH for 4 hours under nitrogen at room temperature. After hydrolysis, the

mixture was acidified to pH 2 to precipitate any proteineaous materials. The supernatant was collected and centrifuged at 3000xg for 10 min at 5 °C to remove any solid materials. After centrifugation, the supernatant was used for phenolic determination as described above.

G. Assessment of the effect of sinapine bisulfate and sinapic acid on the color of protein isolates

The starting material used to examine the effects of added phenolics was an isolate prepared using the AAP method (Owen et al., 1971). The protein isolate was extracted with methanol several times until no sinapine or sinapic acid were detected. Twenty mg of sinapine bisulfate or sinapic acid were added to and throughly mixed with 2 g of the phenolic free protein isolate. These mixtures were used to conduct two experiments designed to simulate processing conditions.

a. Simulation of basic extraction with acidic precipitation. Three samples were used in this study: protein isolate containing no phenolics (used as a control); protein isolate containing 1% sinapine bisulfate; and protein isolate containing 1% sinapic acid. For each sample 2 g were dispersed into 100 mL water, the pH adjusted to 10.5 with 1 N NaOH solution and stirred for 1 hour, 5 hours and 24 hours. The pH was readjusted several times during the simulated extraction to maintain a constant value of 10.5. After the simulated extraction, the pH of the mixture was adjusted to pH 4 with 1 M HCl, and the protein precipitates were allowed to settle. The supernatant was removed by decanting. The precipitate was stored in the refrigerator overnight before freeze-drying. After freeze-drying,

the protein isolates were analyzed for color and phenolic contents.

b. Simulation of autoclaving. Using the same samples, 2 g of each were autoclaved for 15, 30 and 45 min, respectively, at 121 °C (250 F) and 0.1 MPa (15 Psi) in an AMSCO Eagle 3000 Series Sterilizer (American Sterilizer Company, Horsham, PA). After autoclaving, the protein isolates were analyzed for color and phenolic content.

H. Statistical analysis

Each reported value was the mean of three replicates. The analysis of variance and Duncan's multiple range test were performed using the SAS System for Windows (version 6.12) statistical procedure.

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RESULTS AND DISCUSSION

A. Effects of extraction procedures on the color of canola protein isolate

The effects of extraction procedures on the color of canola protein isolates are shown in Table 6.1. Basic extraction with acidic precipitation (BAP) produced protein isolates with the lowest L values while aqueous NaCl extraction with acidic precipitation (AAP) method produced a protein isolate with the highest L value and therefore the brightest product. Based on a comparison of the a values, basic extraction intensified the red color component (positive a values) while aqueous NaCl extraction intensified the green component (negative a values). Based on the b values, the most intense yellow color was obtained for the extraction at pH 8.5 while the least intense was with the PMM procedure. Overall, the appearance of the pH 10 isolate could be described as dark brown, that of the pH 8.5 isolate deep yellow, that of PMM isolate light brown and that of the AAP isolate grey. It is clear, therefore, that protein isolated using the basic extraction with acidic precipitation was the darkest while the isolates prepared using aqueous NaCl extraction with acidic precipitation were lighter. The AAP and PMM procedures differ primarily in the method of protein precipitation. The precipitation of the former was rapid, and immediately followed the extraction. On the other hand, the precipitation by dilution required in the PMM procedure took much longer and the extract had to be concentrated using ultrafiltration prior to precipitation. The longer time required for concentration and precipitation may have caused the color to be darker than the AAP

sample.

Table 6.1. Effect of processing condition on the color of canola protein isolate [a]

Procedure [b]	Color			Protein content Yield % [c] (%Nx5.8)	
	L	a	b		
AAP	71.7±0.3^A	-2.5±0.7^C	14.6±0.9^B	78.17±0.94^A	2.6
PMM	59.7±1.0^B	-1.3±0.3^B	10.1±0.5^C	78.11±0.17^A	1.4
BAP (pH 8.5)	48.8±1.2^C	0.8±0.4^A	19.9±0.6^A	66.05±0.14^B	1.8
BAP (pH 10)	34.5±0.7^D	0.6±0.2^A	12.1±0.4^D	64.60±0.17^C	3.8

[a] Three replicates±SD. Column values with the same letters were not significantly different ($p \leq 0.05$).

[b] AAP = aqueous extraction with acidic precipitation, BAP = basic extraction with acidic precipitation, PMM = protein micellar mass (aqueous extraction with precipitation by dilution).

[c] Yield is based on a comparison of the weight of the protein isolate recovered to the original weight of meal used.

The yield of the protein isolate from the pH 10 BAP method was the highest, with a yield from the AAP and PMM procedures somewhat lower (Table 6.1). However, the protein content of this basic protein isolate was only 64.6%, lower in comparison with protein contents of approximately 78.2% for the AAP and PMM isolates. This indicated that the high yield obtained with the basic extraction at pH 10 may be caused by extraction of more

impurities under basic condition. Basic extraction at pH 8.5 caused both a low protein content (66.05%) and a low yield (1.8%) for the isolate. This is not always the case as Keshavarz et al. (1977) reported a high protein content for the protein isolate from basic extraction.

Results from the analysis of soluble phenolics of the above protein isolates are given in Table 6.2. The total soluble phenolic content of the BAP isolate was about 3-5 times higher than that of AAP and PMM isolates, when comparing the values obtained using HPLC or the Folin-Ciocalteu reagent method. In addition, proteins extracted with alkali had sinapine contents more than twice those of the AAP and PMM isolates. Therefore, basic conditions extracted more phenolics and higher levels of impurities (Table 6.1). It is also possible that some pigments of seed origin or pigments developed during oilseed processing would be easily extracted under basic condition and this could partly account for color observed for these protein isolates. Sinapic acid levels were similar for all four isolates.

The total phenolic contents determined by Folin-Ciocalteu's reagent were much higher than those determined by HPLC. However, the trends of the results for both methods were similar.

Qualitative results of the HPLC chromatograms of the various methanol extracts from the four different protein isolates were given in Fig.6.1 (a-d). As noted above, the sinapine content of the BAP isolates (Fig.6.1c-d) was clearly much higher than those of the PMM and AAP isolates (Fig.6.1a-b). The chromatograms of the AAP isolate and BAP isolates also showed many small unidentified peaks before the elution time of 6 min. It was possible that the addition of acid or alkali during the isolation produced new substances.

Table 6.2. Soluble phenolic contents of protein isolate [a]

Procedure [d]	Soluble phenolics (mg/g)			
	Sinapine [b]	Sinapic acid [b]	Total [b]	Total [c]
AAP	0.45 ± 0.12^B	0.18 ± 0.07^A	2.95 ± 0.79^C	5.67 ± 0.22^C
PMM	0.74 ± 0.29^B	0.20 ± 0.04^A	2.22 ± 0.13^C	6.84 ± 0.98^C
BAP (pH 8.5)	1.62 ± 0.24^A	0.21 ± 0.03^A	8.22 ± 0.26^B	14.03 ± 0.98^B
BAP (pH 10)	1.74 ± 0.23^A	0.23 ± 0.02^A	10.42 ± 0.79^A	17.66 ± 1.23^A

[a] Three replicates$\pm$SD. Column values with the same letters were not significantly different ($p \leq 0.05$). [b] Determined by HPLC. [c] Determined by Folin-Ciocalteu's reagent method. [d] Abbreviations as in Table 6.1.

B. Effect of autoclaving on the color of canola flour

The effect of autoclaving on the color of the whole seed and dehulled canola flour is shown in Table 6.3. The lightest (highest L value) was obtained from the untreated dehulled flour. The inclusion of hull component in the whole seed flour produced a darker flour. The effect of autoclaving, however, was the same for the two flours. There was a decrease in the L value and an increase in the a value as the time of autoclaving increased. The b value did not change significantly. Therefore, the flours darkened and became more red as a result of the autoclaving treatment. While the dehulled flour was still lighter than the whole seed flour after 45 minutes of autoclaving, the difference was not as great as was seen for the untreated seed.

Fig.6.1a-d. HPLC chromatograms of methanol extracts from PMM (a, 100 mg PMM/5 mL methanol, 4 μL injected), AAP (b, 50 mg AAP/12.5 mL methanol, 5 μL injected), and BAP pH 8.5 (c, 50 mg BAP/12.5 mL methanol, 5 μL injected) and BAP pH 10 (d, 50 mg BAP/12.5 mL methanol, 5 μL injected) isolates. Peak identifications: 1. sinapic acid, 2. sinapine, other peaks were unidentified.

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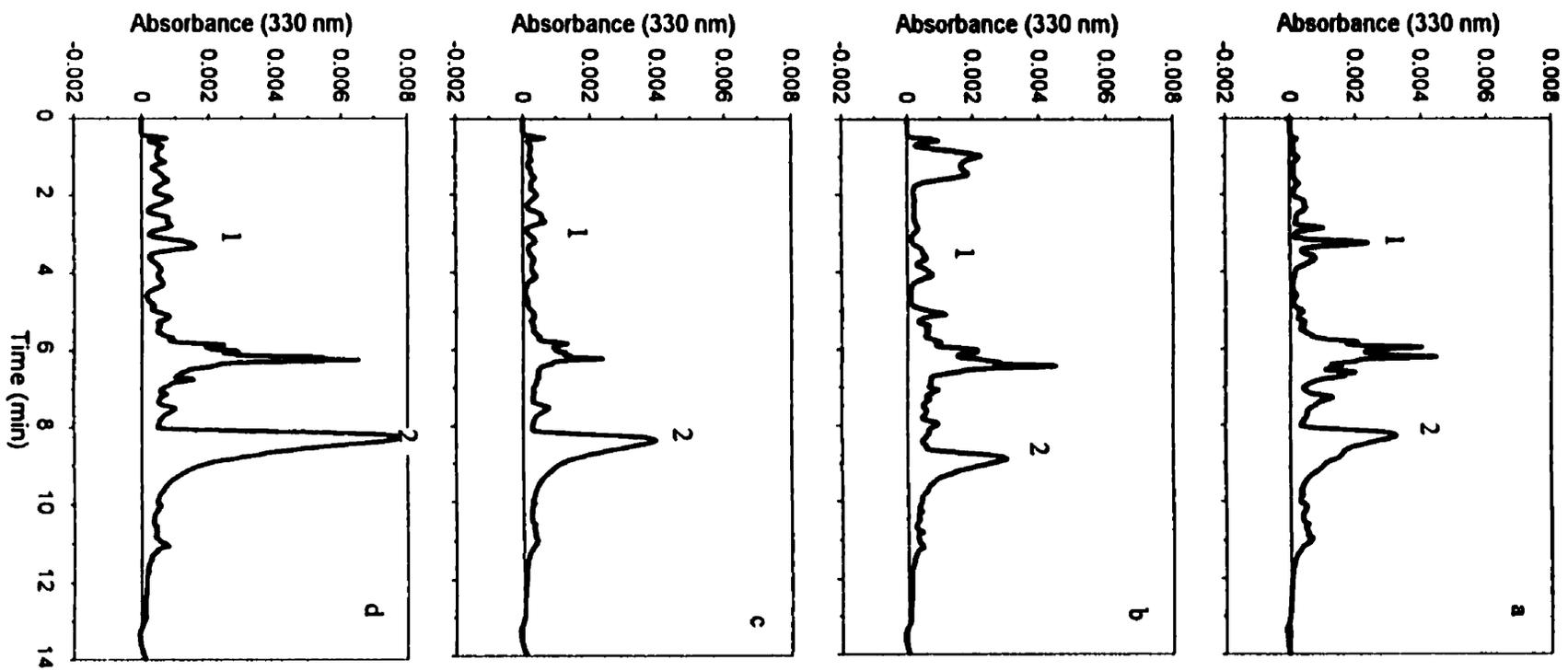
Table 6.3. Effect of time on the color of canola flour during autoclaving [a]

Time (min)	Color		
	L	a	b
	Whole seed flour		
	L	a	b
0	70.2 ± 0.1^A	2.1 ± 0.1^A	17.3 ± 0.1^A
15	58.9 ± 0.2^B	5.1 ± 0.1^B	17.3 ± 0.1^A
30	54.8 ± 0.4^C	7.0 ± 0.1^C	17.2 ± 0.1^A
45	51.3 ± 0.3^D	8.3 ± 0.1^D	17.0 ± 0.2^A
	Dehulled canola flour		
	L	a	b
0	79.2 ± 0.1^A	0.5 ± 0.1^A	19.7 ± 0.7^A
15	63.0 ± 0.1^B	4.9 ± 0.2^B	19.3 ± 0.1^A
30	58.4 ± 0.3^C	6.7 ± 0.1^C	19.4 ± 0.1^A
45	54.5 ± 0.8^D	8.2 ± 0.1^D	19.0 ± 0.4^A

[a] Three replicates$\pm$SD. Column values with the same letters were not significantly different ($p\leq0.05$).

Changes of the soluble and insoluble-bound phenolics as a results of autoclaving are shown in Table 6.4. All soluble phenolics were higher in the untreated dehulled flour. Although the presence of hull material gave a darker color (Table 6.3), the hull component has been reported to contain a lower level of phenolic compounds than the kernel (Krygier et al., 1982b). As a result, it is not surprising that the removal of hulls gives a product in which the level of various phenolic compounds is elevated.

The effects of autoclaving were similar for both the whole and dehulled canola flours (Table 6.4). There were significant decreases in the total soluble phenolics (for both HPLC and the Folin-Ciocalteu methods) and in sinapine contents for both flours, and the greatest decrease came during the first 15 minutes of autoclaving. A significant decrease in soluble sinapic acid was not seen with the whole seed flour. Perhaps the lower levels of sinapic acid in the unheated whole seed flour made it more difficult to find significant differences. The levels of sinapic acid for both flours were considerably lower than the sinapine levels.

For both flours, the reduced levels of soluble phenolics were accompanied by an increase in the level of insoluble-bound phenolics, which was also most notable after the first 15 minutes of autoclaving. These results indicate that during autoclaving some soluble phenolics may have been converted to insoluble-bound phenolics. The mechanism of this conversion will require further investigation. It has been reported previously that heat treatments can reduce the sinapine content but increase the lignan content of rapeseed flour.(Jensen et al., 1990).

Table 6.4. Effect of autoclaving time on the soluble and insoluble-bound phenolic content in canola flour [a]

Time (min)	Soluble phenolics (mg/g)				Insoluble-bound phenolics (mg/g)		
	Sinapine [b]	Sinapic acid [b]	Total [b]	Total [c]	Sinapic acid [b]	Total [b]	Total [c]
			Whole canola flour				
0	12.09 ± 0.22^A	0.49 ± 0.11^A	32.50 ± 1.13^A	34.60 ± 0.61^A	0.08 ± 0.02^B	1.71 ± 0.06^A	4.75 ± 0.69^B
15	10.97 ± 0.60^B	0.43 ± 0.03^A	21.15 ± 1.10^B	25.89 ± 1.68^B	0.12 ± 0.02^{AB}	1.82 ± 0.17^A	6.24 ± 0.81^A
30	10.59 ± 0.74^B	0.42 ± 0.05^A	20.42 ± 2.30^B	25.20 ± 2.09^{BC}	0.13 ± 0.03^A	1.85 ± 0.12^A	6.95 ± 0.78^A
45	9.58 ± 0.43^C	0.40 ± 0.03^A	18.56 ± 0.93^B	22.70 ± 1.33^C	0.14 ± 0.03^A	1.92 ± 0.05^A	7.60 ± 0.78^A
			Dehulled canola flour				
0	15.84 ± 0.46^A	0.73 ± 0.10^A	42.84 ± 1.77^A	44.08 ± 2.18^A	0.08 ± 0.02^B	1.05 ± 0.08^B	3.24 ± 0.54^B
15	12.75 ± 0.91^B	0.43 ± 0.03^B	30.18 ± 1.84^B	38.75 ± 1.38^B	0.64 ± 0.08^A	1.80 ± 0.07^A	6.72 ± 1.42^A
30	12.31 ± 1.06^B	0.31 ± 0.06^{BC}	29.30 ± 2.77^B	37.32 ± 1.48^{BC}	0.65 ± 0.08^A	1.87 ± 0.10^A	7.34 ± 0.78^A
45	11.19 ± 1.00^B	0.29 ± 0.06^C	26.40 ± 2.68^B	33.93 ± 2.24^C	0.72 ± 0.04^A	1.93 ± 0.15^A	7.95 ± 0.72^A

[a] Three replicates±SD. Column values within type of each flour with the same letters were not significantly different ($p \leq 0.05$).

[b] Determined by HPLC.

[c] Determined by Folin-Ciocalteu's reagent method.

Representative chromatograms of the methanol extracts from dehulled canola flour before and after autoclaving are given in Fig.6.2a-b, demonstrating the decrease in peak height with autoclaving. A comparison of the 95% ethanol (ethanol/water, 95/5, v/v) extracts from whole seed flour and industrial meal (Fig.6.3a-b) shows that the peak heights for the industrial meal were much lower than those for the whole seed flour (Fig.6.3a-b). This could be a result of the effect of autoclaving demonstrated above.

C. Effects of sinapine bisulfate and sinapic acid on the color of canola protein isolate during simulated basic extraction

The effects of added sinapine bisulfate and sinapic acid on the color of canola protein isolate during the simulated basic extraction are shown in Table 6.5a. After one hour of simulated basic extraction, the L value of the control was significantly lower than those of the isolates containing 1% sinapine bisulfate and 1% sinapic acid ($p \leq 0.05$). After 5 hours of simulated basic extraction, the L value of the control decreased only slightly to 64.6 while those of the isolates with 1% sinapine bisulfate and 1% sinapic acid continued to decrease so that the L value of the isolate containing 1% sinapine bisulfate was significantly higher but the L value for the isolate containing 1% sinapic acid was not significantly different from the control ($p \leq 0.05$).
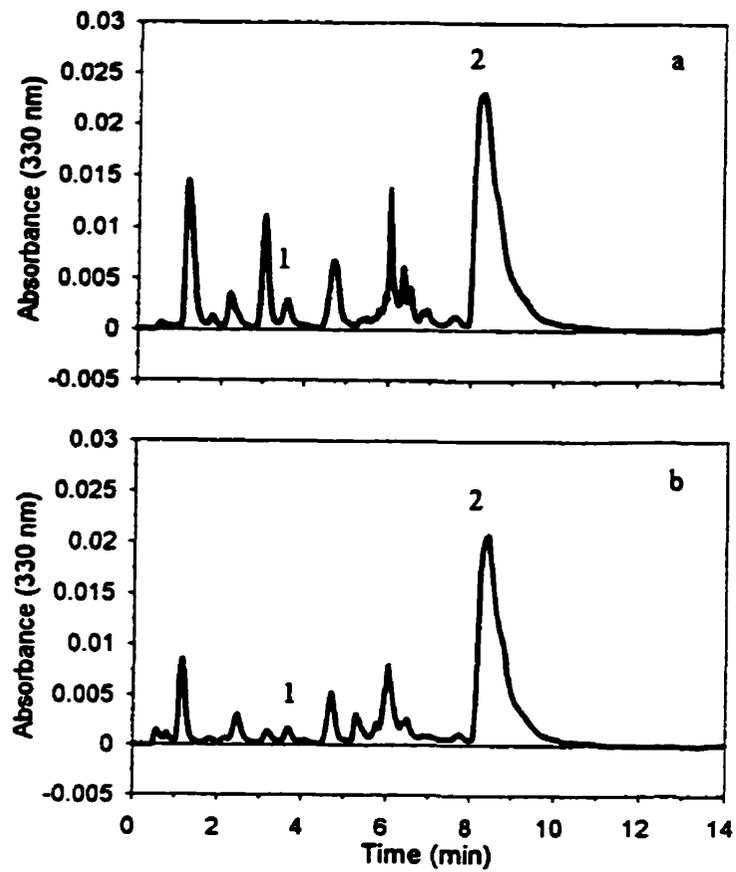
Fig.6.2a-b. HPLC chromatograms of 100% methanol extracts from kernel flour before (a) and after (b) autoclaving. Peak identifications: 1. sinapic acid, 2. sinapine, other peaks were unidentified.
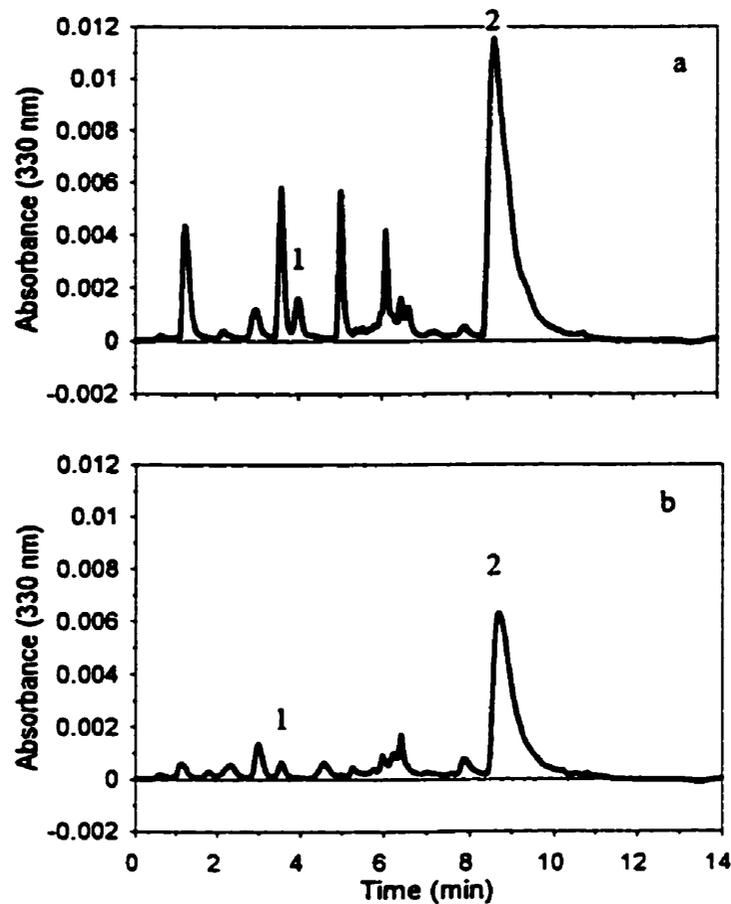
Fig.6.3a-b. HPLC chromatograms of 95% ethanol extracts from whole seed flour (a) and industrial meal (b). Peak identifications: 1. sinapic acid, 2. sinapine, other peaks were unidentified.

Table 6.5a. Effect of sinapine bisulfate and sinapic acid on the color of canola protein isolate during basic extraction[a].

Phenolic added [b]	Color		
	L	a	b
	0 hour		
Control	69.6±0.5	-1.7±0.1	12.9±0.2
	1 hour		
0	64.0±0.3[C]	-1.7±0.2[A]	12.7±0.2[A]
1% SPN	67.7±0.2[A]	-2.2±0.1[B]	13.1±0.6[A]
1% SA	66.5±0.3[B]	-1.6±0.1[A]	12.4±0.4[A]
	5 hours		
0	64.6±0.2[B]	-1.6±0.1[A]	11.7±0.2[A]
1% SPN	65.6±0.2[A]	-1.8±0.2[A]	11.7±0.2[A]
1% SA	64.6±0.2[B]	-1.7±0.1[A]	11.4±0.4[A]
	24 hours		
0	63.3±0.2[C]	-1.6±0.1[A]	10.5±0.2[A]
1% SPN	64.5±0.5[B]	-1.6±0.1[A]	10.3±0.1[A]
1%SA	66.6±0.1[A]	-1.5±0.2[A]	10.2±0.2[A]

[a] Three replicates±SD. Column values with the same letters were not significantly different (p≤0.05). [b] SPN = Sinapine bisulfate, SA = Sinapic acid, 0 = No phenolics.

After 24 hours, the L value of the control decreased to 63.3 while those of the isolates with 1% sinapine bisulfate and 1% sinapic acid were significantly higher ($p \leq 0.05$) with the highest value associated with the isolate containing 1% sinapic acid. With the exception of

a decrease in the a value for the isolate containing 1% sinapine bisulfate after 1 h, the a and b values of the isolates with and without phenolics were not significantly different ($p \leq 0.05$). These results indicated that added sinapine bisulfate and sinapic acid did not promote darkening and, in fact, slowed down the darkening process (increased L value) of protein isolates particularly during the first hour of the exposure to alkali. Neither phenolic had any negative effect on the color of the canola protein isolate over the 24-hour period of the extraction period.

The effect of time on the color of canola protein isolate during basic extraction can be evaluated more closely by reorganization of the same data (Table 6.5b). For the control sample with no phenolics, there were significant decrease in the L and b value from zero to 24 hours with the greatest change occurring in the first 1-5 hours. The a value remained unchanged. Similar trends were seen with the isolates containing 1% sinapine bisulfate and 1% sinapic acid.

In addition to the significant effect of time and the presence of either sinapine bisulfate and sinapic acid, results from the analysis of variance (Appendix 6.5d, e) indicated a significant interaction between time and the presence of either phenolic in terms of the L value and a significant interaction between time and the presence of sinapine bisulfate in terms of the a value. This demonstrates that the relationship between the L value and the addition of phenolic (or a value and the addition of sinapine bisulfate) differed at the various times of exposure to the basic condition.

Table 6.5b. Effect of time on the color of canola protein isolate during basic extraction [a]

Time (h)	Color		
	L	a	b
Control (no phenolics)			
0	69.6±0.5[A]	-1.7±0.1[A]	12.9±0.2[A]
1	64.0±0.3[C]	-1.7±0.2[A]	12.7±0.2[A]
5	64.6±0.2[B]	-1.6±0.1[A]	11.7±0.2[B]
24	63.3±0.2[D]	-1.6±0.1[A]	10.5±0.2[C]
1% Sinapine bisulfate			
0	69.6±0.5[A]	-1.7±0.1[A]	12.9±0.2[A]
1	67.7±0.2[B]	-2.2±0.1[B]	13.1±0.6[A]
5	65.6±0.2[C]	-1.8±0.2[A]	11.7±0.2[B]
24	64.5±0.5[D]	-1.6±0.1[A]	10.3±0.1[B]
1% Sinapic acid			
0	69.6±0.5[A]	-1.7±0.1[A]	12.9±0.2[A]
1	66.5±0.3[B]	-1.6±0.1[A]	12.4±0.4[A]
5	64.6±0.2[C]	-1.7±0.1[A]	11.4±0.4[B]
24	66.6±0.1[B]	-1.5±0.2[A]	10.2±0.2[C]

[a] Three replicates±SD. Column values with the same letters were not significantly different ($p \leq 0.05$).

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The effect of time of exposure to basic conditions on the retained soluble phenolic content in canola protein isolate is shown in Table 6.6. For the sample to which no phenolics had been added, the content of sinapine and sinapic acid increased slightly after 24 h but the total soluble phenolic content determined by HPLC and the Folin-Ciocalteu method decreased. For the isolate with 1% sinapine bisulfate added, the sinapine level and the total phenolic content decreased significantly during the 24 hours of simulated basic extraction. At the same time, there was a significant increase in the sinapic acid content.

The greatest loss of sinapine occurred during the protein precipitation step which followed the extraction in the simulation exercise. At this point, sinapine was removed in the discarded supernatant. As a result the greatest changes were observed between zero and 1 h hour, when this precipitation step was first included. The hydrolysis of sinapine to sinapic acid could also account for some losses of sinapine that occurred after the first hour of the extraction. A small amount of thomasidioic acid was also detected, which may also have derived from sinapine via sinapic acid.

Similar trends were found for the isolate with 1% sinapic acid. Both sinapic acid and the total phenolic content decreased significantly after 24 h of extraction. A small amount of sinapine was detected after 24 h, which was probably released from the seed. As was the case with sinapine, the greatest losses of sinapic acid probably occurred during protein precipitation. A small amount of thomasidioic acid detected after 5 h and 24 h of extraction, has been reported to be produced from sinapic acid (Rubino et al., 1995).

In all three samples, the total phenolic content determined by Folin-Ciocalteu's

reagent method was much higher than that determined by the HPLC method. However, the trends of the results were similar for both methods.

Table 6.6. Effect of time on the retained soluble phenolic content in canola protein isolate during basic extraction [a]

Time (min)	Soluble phenolics (mg/g)			
	Sinapine [b]	Sinapic acid [b]	Total [b]	Total [c]
No phenolics (control)				
0	0.04 ± 0.03^B	0^C	0.98 ± 0.20^A	5.9 ± 0.38^A
1	0^C	0^C	----	—
5	0^C	0.01 ± 0.003^B	0.11 ± 0.03^B	0.84 ± 0.06^B
24	0.10 ± 0.03^A	0.03 ± 0.01^A	0.23 ± 0.03^B	1.14 ± 0.07^B
1% Sinapine bisulfate				
0	8.70 ± 0.43^A	0^C	11.34 ± 0.41^A	15.70 ± 1.2^A
1	3.48 ± 0.26^B	0^C	3.85 ± 0.36^B	5.51 ± 0.22^B
5	2.73 ± 0.18^C	0.43 ± 0.03^B	3.89 ± 0.39^B	5.84 ± 0.37^B
24	1.74 ± 0.15^D	0.56 ± 0.04^A	$3.72\pm0.09^{d\,B}$	5.20 ± 0.85^B
1% Sinapic acid				
0	0^B	10.52 ± 0.62^A	11.70 ± 0.98^A	14.3 ± 1.12^A
1	0^B	4.63 ± 0.57^B	4.73 ± 0.33^B	5.92 ± 0.3^B
5	0^B	3.78 ± 0.33^B	$5.05\pm0.25^{d\,B}$	6.92 ± 0.51^B
24	0.03 ± 0.01^A	1.81 ± 0.12^C	$4.95\pm0.34^{d\,B}$	5.83 ± 0.13^B

[a] Three replicates$\pm$SD. Column values with the same letters (A, B, C, or D) were not significantly different ($p\leq0.05$). [b] Determined by HPLC. [c] Determined by Folin-Ciocalteu's reagent method. [d] A small amount of thomasidioic acid was detected. [e] At zero time measurements on original isolate with no extraction or protein precipitation.

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The chromatograms of methanol extracts of the retained phenolics at different reaction times are given in Fig.6.4a-c.

D. Effects of sinapine bisulfate and sinapic acid on the color of canola protein isolate during autoclaving

The effects of added sinapine bisulfate and sinapic acid on the color of canola protein isolate during autoclaving are shown in Table 6.7a. Autoclaving had a significant effect on the color of canola protein isolate even when no phenolics were added. The greatest changes occurred during the first 15-min autoclaving with the L value decreasing and the a and b values increasing. The sample containing 1% sinapine bisulfate was consistently darker (lower L value) than either the control or the isolate containing 1% sinapic acid. The b value for the sample containing 1% sinapine bisulfate was not significantly different from the sample with no added phenolics. The sample containing 1% sinapic acid, on the other hand, had the L value the same as or slightly higher (45 min) than the control. Also the a values were similar except at 15 min where the 1% sinapic acid sample was slightly less red. The b values for the 1% sinapic acid sample, however, were consistently higher than the control or 1% sinapine bisulfate sample. This increase in the yellow component is consistent with previous findings which indicated sinapic acid could produce color compounds during autoclaving (Cai et al., 1999a).
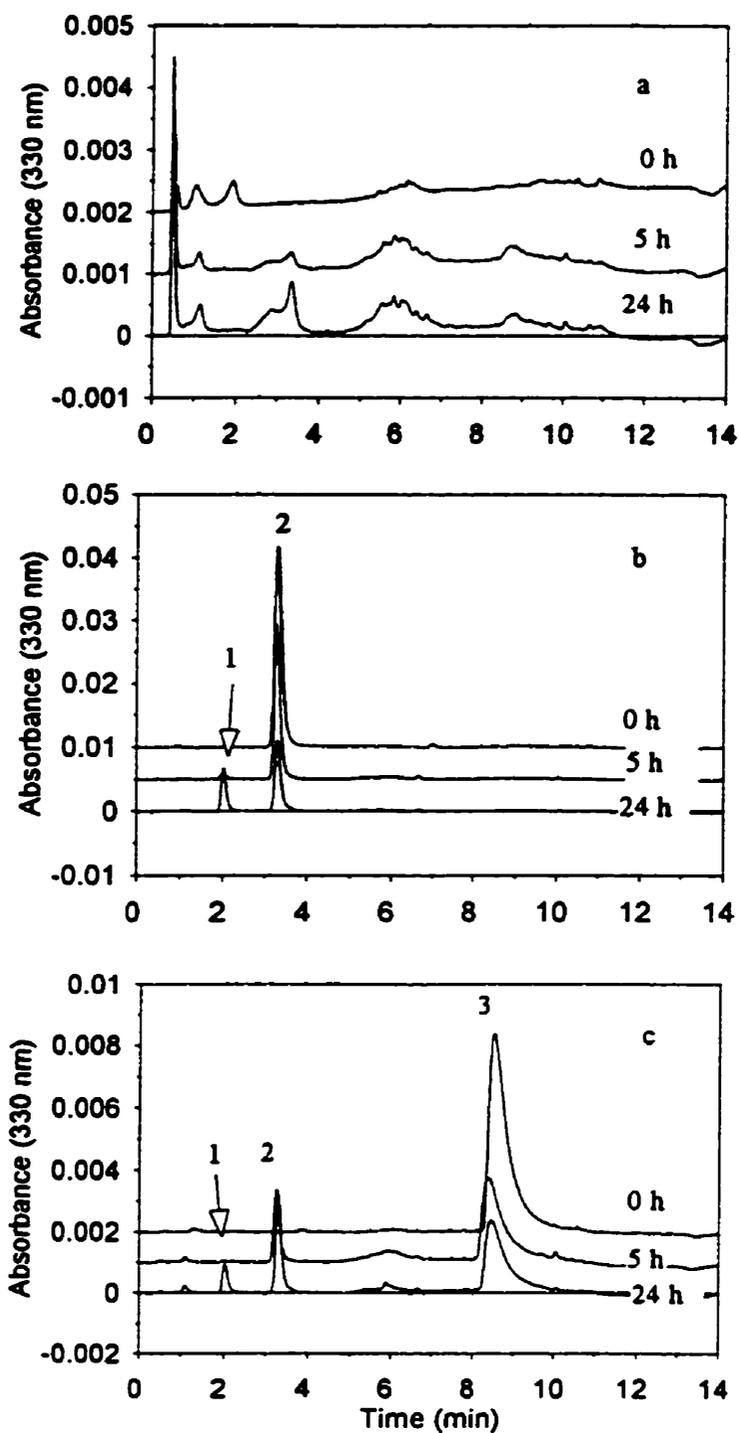
Fig.6.4a-c. HPLC chromatograms of methanol extracts from control isolate (a), isolate with 1% sinapic acid (b) and isolate with 1% sinapine (c) after basic extraction. Peak identifications: 1. Thomasidioic acid, 2. sinapic acid, 3. sinapine, other peaks unidentified.

Table 6.7a. Effects of sinapine bisulfate and sinapic acid during autoclaving on the color of canola protein isolate [a]

Phenolic added [b]	Color		
	L	a	b
	0 min (control)		
	69.6±0.5	-1.7±0.1	12.9±0.2
	15 min		
0	52.3±0.3^A	0.5±0.1^B	15.1±0.2^B
1%SPN	51.3±0.3^B	0.7±0.1^A	15.2±0.3^B
1%SA	52.6±0.2^A	0.2±0.1^C	16.1±0.1^A
	30 min		
0	50.3±0.7^A	-0.2±0.1^B	14.2±0.5^B
1%SPN	48.0±0.3^B	0.2±0.2^A	14.0±0.1^B
1%SA	50.3±0.3^A	-0.2±0.1^B	15.7±0.1^A
	45 min		
0	48.4±0.2^B	0.7±0.1^{AB}	14.7±0.1^B
1%SPN	47.1±0.4^C	1.0±0.3^A	14.4±0.3^B
1%SA	49.8±0.1^A	0.5±0.1^B	15.9±0.2^A

[a] Three replicates±SD. Column values with the same letters were not significantly different ($p \leq 0.05$).

[b] SPN = Sinapine bisulfate, SA = Sinapic acid, 0 = No phenolics, i.e., control.

The effect of time during autoclaving on the color of canola protein isolate with added sinapine bisulfate and sinapic acid is more easily demonstrated by rearranging the same data (Table 6.7b).

Table 6.7b. The effect of time during autoclaving on the color of canola protein isolate [a]

Time (h)	Color		
	L	a	b
	Control (no phenolics)		
0	69.6 ± 0.5^A	-1.7 ± 0.1^D	12.9 ± 0.2^C
15	52.3 ± 0.3^B	0.5 ± 0.1^B	15.1 ± 0.2^A
30	50.3 ± 0.7^C	-0.2 ± 0.1^C	14.2 ± 0.5^B
45	48.4 ± 0.2^D	0.7 ± 0.1^A	14.7 ± 0.1^B
	1% Sinapine bisulfate		
0	69.6 ± 0.5^A	-1.7 ± 0.1^C	12.9 ± 0.2^C
15	51.3 ± 0.3^B	0.7 ± 0.1^A	15.2 ± 0.3^A
30	48.0 ± 0.3^C	0.2 ± 0.2^B	14.0 ± 0.1^B
45	47.1 ± 0.4^D	1.0 ± 0.3^A	14.4 ± 0.3^B
	1% Sinapic acid		
0	69.6 ± 0.5^A	-1.7 ± 0.1^D	12.9 ± 0.2^C
15	52.6 ± 0.2^B	0.2 ± 0.1^B	16.1 ± 0.1^A
30	50.3 ± 0.3^C	-0.2 ± 0.1^C	15.7 ± 0.1^B
45	49.8 ± 0.1^D	0.5 ± 0.1^A	15.9 ± 0.2^B

[a] Three replicates±SD. Column values with the same letters were not significantly different ($p\leq0.05$).

It is also clearly demonstrated from these data that the greatest changes take place during the first 15 min of autoclaving. While the samples continue to darken after this time (L values lower), the a and b values do not change. The a values reach their highest values after 45 min but the b values for all three samples are at their highest values after 15 min. It would appear that changes in the later stages are altered from those in the early stages of autoclaving. This is most clearly demonstrated by the changes in the yellow component (b value).

Results from the analysis of variance showed that the interaction effect between time and the presence of 1% sinapine bisulfate or 1% sinapic acid was significant for the L values but not significant for a and b values (Appendix 6.7d-e).

The effect of autoclaving time on the soluble and insoluble-bound phenolic contents of canola protein isolates containing added phenolic is given in Table 6.8. For the soluble phenolic content of the control (no added phenolics), sinapine content increased significantly during autoclaving, particularly during the first 15 min. On the other hand, sinapic acid was detected only in small amounts at 15 min and 45 min. The total soluble phenolic content determined by either HPLC or Folin-Ciocalteu method decreased significantly during the 45-min autoclaving, although the levels were considerably higher with the Folin-Ciocalteu method. For the insoluble-bound phenolic content, sinapic acid and the total phenolic content increased significantly during the 45-min autoclaving.

Table 6.8. Effect of autoclaving time on the soluble and insoluble-bound phenolic contents in the mixture of phenolic-protein isolate$_a$

Time	Soluble phenolics (mg/g)				Insoluble-bound phenolics (mg/g)		
	Sinapine[b]	Sinapic acid[b]	Total[b]	Total[c]	Sinapic acid[b]	Total[b]	Total[c]
0 Phenolics							
0	0.04 ± 0.03^C	0^B	0.98 ± 0.2^A	5.90 ± 0.38^A	0^C	0.10 ± 0.01^C	3.10 ± 0.17^B
15	0.34 ± 0.03^A	0.03 ± 0.01^A	0.73 ± 0.08^B	5.10 ± 0.21^B	0.01 ± 0.003^B	0.21 ± 0.03^B	4.32 ± 0.59^A
30	0.10 ± 0.02^B	0^B	0.45 ± 0.04^C	4.90 ± 0.35^{BC}	0.01 ± 0.002^B	0.25 ± 0.02^{AB}	4.42 ± 0.20^A
45	0.10 ± 0.03^B	0.010 ± 0.001^B	0.22 ± 0.02^D	4.51 ± 0.15^C	0.02 ± 0.008^A	0.29 ± 0.05^A	4.51 ± 0.31^A
1% Sinapine bisulfate							
0	8.70 ± 0.43^A	0^B	11.34 ± 0.41^A	15.70 ± 1.2^A	0.01 ± 0.01^D	0.15 ± 0.04^C	4.12 ± 0.20^B
15	6.08 ± 0.17^B	0^B	6.85 ± 0.39^B	10.5 ± 0.74^B	0.40 ± 0.04^C	0.72 ± 0.10^B	6.56 ± 0.41^A
30	5.87 ± 0.46^B	0.01 ± 0.01^B	6.50 ± 0.34^B	9.80 ± 0.55^B	0.52 ± 0.03^B	0.83 ± 0.10^B	6.87 ± 0.42^A
45	5.31 ± 0.91^B	0.04 ± 0.01^A	5.52 ± 0.50^C	9.20 ± 0.20^B	0.60 ± 0.03^A	1.30 ± 0.15^A	6.97 ± 0.36^A
1% Sinapic acid							
0	0^C	10.52 ± 0.62^A	11.70 ± 0.98^A	14.30 ± 1.12^A	0.010 ± 0.004^D	0.010 ± 0.003^D	4.21 ± 0.20^C
15	0^C	6.49 ± 0.34^B	6.61 ± 0.37^B	10.10 ± 1.03^B	0.34 ± 0.02^C	0.75 ± 0.08^C	6.89 ± 0.50^A
30	0.06 ± 0.02^B	3.58 ± 0.14^C	5.12 ± 0.53^C	8.85 ± 0.33^B	0.60 ± 0.06^B	0.95 ± 0.11^B	7.12 ± 0.39^A
45	0.16 ± 0.05^A	0.87 ± 0.05^D	1.98 ± 0.13^D	5.01 ± 1.37^C	0.75 ± 0.04^A	1.70 ± 0.07^A	6.11 ± 0.29^B

[a] Three replicates±SD. Column values within each level/type of phenolic with the same letters were not significantly different ($p\leq0.05$). [b] Determined by HPLC. [c] Determined by Folin-Ciocalteu's reagent method.

For the soluble phenolic content of the isolate containing 1% sinapine bisulfate, the sinapine level dropped significantly over 45 min. Sinapic acid was detected after 30 min. The total phenolic content decreased significantly. Both the total insoluble-bound phenolic content and insoluble-bound sinapic acid increased over the same period.

For the soluble phenolic content of the isolate containing 1% sinapic acid, sinapic acid and total phenolic content decreased over 45 min, while there was a slight increase in sinapine content. As was the case for the other two samples, the insoluble-bound phenolic content, including sinapic acid and total phenolic content increased significantly over 45 min.

For all the samples, the total phenolic contents determined by Folin-Ciocalteu's reagent method were again much higher than those determined by HPLC. However, the trends in terms of the effect of autoclaving in the presence of different phenolics were similar.

Changes in phenolic contents in the mixtures of phenolic-protein isolates followed a similar trend as was seen previously for canola flour.

The decrease in soluble phenolics and the resulting increase in insoluble-bound phenolics corresponded to the darkening of the protein isolate, including the observation that the greatest changes occurred during the first 15 min. In addition, the samples to which the phenolics had been added showed unique changes in color with more sinapine bisulfate resulting in a darker color and more sinapic acid producing a yellow color. While the increase in yellow color was shown to occur for sinapic acid when exposed to autoclaving, sinapine bisulfate alone did not produce a color change. In this situation, a reaction between sinapine bisulfate and some other component in the protein isolate is probably responsible for the

reaction.

From this study, it was concluded that basic extraction with acidic precipitation produced a much darker protein isolate than did aqueous NaCl extraction with acidic precipitation or precipitation by dilution. While the sinapine content of the isolate prepared by basic extraction was elevated in comparison with the other isolates, the addition of sinapine bisulfate and sinapic acid to the isolate prior to the exposure to alkaline conditions of up to 24 h indicated that added sinapine bisulfate and sinapic acid did not have a negative effect on the color of the canola protein isolate. These phenolics may not be major factors responsible for the dark color of the protein isolate produced using basic extraction procedures. On the other hand, autoclaving darkened the color of both whole seed flour and dehulled canola flour. In both flours, soluble phenolic content decreased while insoluble-bound phenolic content increased as a result of autoclaving. The addition of sinapine bisulfate was shown to decrease the L value (color darkened) of the protein isolate while sinapic acid addition increased the b value (yellow intensified) during the autoclaving. It should also be noted that autoclaving darkened the control which contained minimum levels of phenolics and no added sinapine bisulfate nor sinapic acid.

CONCLUSIONS

The objectives of this thesis have been focused on the role of the phenolics in color darkening during autoclaving and alkaline treatment, since these two conditions have been associated with the dark color of canola meal and protein isolate. A primary objective has been the examination of the color and structural changes in pure sinapic acid and sinapine under these two conditions. Autoclaving caused an immediate color darkening in sinapic acid aqueous solutions but did not have any negative effect on sinapine aqueous solution. This suggested that sinapic acid was more susceptible to color darkening than sinapine. However, the level of sinapic acid (0.04%) in canola meal is much lower than that of sinapine (1.2%) and it would be expected that changes in sinapine would have a greater influence on the color of canola products. It is possible that the color darkening mechanism of these phenolics in the meal is different from those in pure phenolic systems.

When exposed to alkali, the sinapic acid aqueous solution progressed from yellow-green to brown color as air oxidation continued and produced colored substances including the 2,6-dimethoxy-p-benzoquinone. This colored substance, however, was detected only after 169 hours, a time much longer than that normally required for protein isolation. Air oxidation of sinapine occurred at an even slower rate than for sinapic acid. According to the reaction rate constants of sinapic acid and sinapine, air oxidation of the latter is over ten time slower than the former. These results suggested that the dark color of protein isolates produced from alkaline extraction with acidic precipitation may not be caused by sinapic acid or sinapine,

unless these phenolics behaved differently in terms of color development in a meal system.

The observations and suggestions resulting from the data on the pure phenolic systems were confirmed by conducting experiments in phenolic-protein systems where the effect of sinapic acid and sinapine on the color of canola protein isolate was investigated. During autoclaving, the presence of 1% added sinapine bisulfate resulted in a slight increase in the darkness of the protein isolate. The addition of 1% sinapic acid, on the other hand, increased the yellow intensity of the isolate. While the results of the addition of sinapic acid to a protein isolate appeared to duplicate the result for pure sinapic acid, the result of the addition of sinapine bisulfate resulted in a darkening that had not been apparent with the pure sinapine. Interactions between sinapine and proteins may account for some color darkening for the system.

Sinapic acid and sinapine, when exposed to alkali with protein isolate, on the other hand, did not cause any additional darkening for the color of the protein isolate. In fact they appeared to have a positive effect by slowing down the darkening process. Overall, it can be concluded that the dark color of protein isolate resulting from alkaline isolation is not caused by changes in sinapic acid or sinapine. Therefore, further work should focus on other phenolics or other components that may cause the dark color.

SIGNIFICANT FINDINGS IN THIS THESIS

1. A color change was found in sinapic acid aqueous solution during autoclaving. This appeared to be the first report on color reactions for canola phenolics during autoclaving. These reactions produced several colored compounds. A yellow substance, syringaldehyde, was identified among the colored compounds.

2. Consistent with the above finding, sinapic acid added to a canola protein isolate resulted in an increase in the intensity of yellow color during autoclaving.

3. Sinapine bisulfate alone was not affected by autoclaving and caused no color changes to its aqueous solutions. However, the presence of sinapine bisulfate did cause a darkening for canola protein isolate during autocalving, suggesting that the color darkening mechanism for sinapine in pure sinapine bisulfate aqueous solutions may be different from that of sinapine in the sinapine bisulfate - protein systems.

4. Alkaline air oxidation of sinapic acid and sinapine bisulfate were first order reactions. Alkaline air oxidations of sinapic acid were more than ten times faster than alkaline hydrolysis and air oxidations of sinapine.

5. Colored products resulted from alkaline air oxidation of sinapic acid. While 2,6-dimethoxy-p-benzoquinone was a strong yellow substance, thomasidioic acid showed little color at neutral pH and showed yellowish green at pH 10. 6-hydroxy-5,7-dimethoxy-2-naphthoic acid showed little color at pH 7 - 10.

6. Air oxidation of sinapic acid and sinapine was so slow that it took more time to produce the colored substances than that normally required for protein isolation. Therefore,

neither phenolic had a negative effect on the darkening of canola protein isolate during alkaline isolation.

7. A rapid HPLC method for the simultaneous determination of sinapine and sinapic acid has been developed.

FUTURE RECOMMENDATIONS

1. An investigation is needed to elucidate the undetermined colored substances during the autoclaving of a sinapic acid aqueous solution.

2. Work is needed to identify the colored substances associated with the alkaline air oxidation of sinapine. These colored substances were unidentified although color changes of the sinapine aqueous solution as a function of time have been determined.

3. Unidentified peaks on HPLC chromatograms from determination of sinapine and sinapic acid need to be reexamined.

4. As sinapine and sinapic acid appeared to contribute to the dark color of canola meal and isolate during autoclaving, altering processing to avoid autoclaving should improve the color of canola meal and isolate.

5. Since this research rules out the possible responsibility of sinapine and sinapic acid for the dark color of the canola protein isolate during alkaline isolation, work is needed to identify the possible factors responsible for color darkening under this condition.

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CHAPTER VII. APPENDIXES

Appendix 2.1. Effect of time on the Hunter L a b values of sinapic acid solutions during autoclaving [a].

Time (min)	Hunter color value		
	L	a	b
0	100.0 ± 0.1^A	-5.6 ± 0.1^A	6.2 ± 0.1^C
15	90.9 ± 0.6^B	-8.8 ± 0.3^C	17.5 ± 0.5^B
30	91.2 ± 1.3^B	-7.7 ± 0.5^B	20.4 ± 0.6^A
45	90.7 ± 1.4^B	-7.7 ± 0.3^B	20.9 ± 0.6^A

[a] Mean of three replicates$\pm$SD. Column values with the same letters were not significantly different ($p \leq 0.05$).

Appendix 2.2. ANOVA of Appendix 2.1. The effect of time on the color of a 100 μg/mL sinapic acid solution during autoclaving.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	183.3375583	61.1125194	62.10	0.0001
Error	8	7.8727333	0.9840917		
Corrected Total	11	191.2102917			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	15.61326667	5.20442222	56.20	0.0001
Error	8	0.74080000	0.09260000		
Corrected Total	11	16.35406667			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	426.4161000	142.1387000	631.70	0.0001
Error	8	1.8000667	0.2250083		
Corrected Total	11	428.2161667			

```
Options linesize=78;
Title 'Autoclaving effect on color of sinapic acid, Table 2-1';
Data;
   Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0          100.0     -5.6      6.2
0          99.9      -5.6      6.1
0          100.0     -5.7      6.2
15         91.1      -8.8      17.6
15         91.4      -9.1      18.0
15         90.2      -8.5      17.0
30         91.0      -7.7      20.1
30         92.5      -7.2      21.0
30         90.0      -8.1      20.0
45         90.9      -7.9      20.9
45         92.0      -7.4      21.5
45         89.2      -7.9      20.3
Proc GLM;
   Classes Time;
   Model L a b = Time;
   Means Time / Duncan;
```

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.749973	50	0.0014	0	0.0065	0.052498	0	0.243741	0
390	0.937628	54.6	0.0042	0.0001	0.0201	0.215017	0.005119	1.029009	0.00546
400	0.991603	82.8	0.0143	0.0004	0.0679	1.174098	0.032842	5.574912	0.03312
410	1.00352	91.5	0.0435	0.0012	0.2074	3.99426	0.110186	19.0439	0.1098
420	1.005743	93.4	0.1344	0.004	0.6456	12.62505	0.375746	60.64534	0.3736
430	1.005991	86.7	0.2839	0.0116	1.3856	24.76159	1.011745	120.8512	1.00572
440	1.00539	104.9	0.3483	0.023	1.7471	36.7336	2.425704	184.2586	2.4127
450	1.004896	117	0.3362	0.038	1.7721	39.52799	4.467768	208.3508	4.446
460	1.00426	117.8	0.2908	0.06	1.6692	34.40217	7.09811	197.4694	7.068
470	1.002709	114.9	0.1954	0.091	1.2876	22.51228	10.48423	148.346	10.4559
480	1.003414	115.9	0.0956	0.139	0.813	11.11787	16.1651	94.54839	16.1101
490	1.003731	108.8	0.032	0.208	0.4652	3.49459	22.71483	50.8026	22.6304
500	1.002322	109.4	0.0049	0.323	0.272	0.537305	35.41825	29.8259	35.3362
510	1.001547	107.8	0.0093	0.503	0.1582	1.004091	54.30728	17.08034	54.2234
520	1.000211	104.8	0.0633	0.71	0.0782	6.63524	74.4237	8.197089	74.406
530	0.999754	107.7	0.1655	0.862	0.0422	17.81997	92.81457	4.543822	92.8374
540	1.001195	104.4	0.2904	0.954	0.0203	30.35399	99.71662	2.121853	99.5976
550	1.001301	104	0.4334	0.995	0.0087	45.13224	103.6146	0.905977	103.48
560	1.002639	100	0.5945	0.995	0.0039	59.60689	99.76258	0.391029	99.5
570	1.007724	96.3	0.7621	0.952	0.0021	73.9571	92.38572	0.203792	91.6776
580	1.011946	95.8	0.9163	0.87	0.0017	88.83018	84.34165	0.164806	83.346
590	1.006839	88.7	1.0263	0.757	0.0011	91.65538	67.60511	0.098237	67.1459
600	1.007972	90	1.0622	0.631	0.0008	96.36011	57.24273	0.072574	56.79
610	1.007937	89.6	1.0026	0.503	0.0003	90.54596	45.42651	0.027093	45.0688
620	1.006203	87.7	0.8544	0.381	0.0002	75.39568	33.62097	0.017649	33.4137
630	1.005072	83.8	0.6424	0.265	0	54.10616	22.31963	0	22.207
640	1.004225	83.7	0.4479	0.175	0	37.64762	14.70939	0	14.6475
650	1.005108	80	0.2835	0.107	0	22.79585	8.603724	0	8.56
660	1.004719	82.2	0.1649	0.061	0	13.61875	5.037862	0	5.0142
670	1.004437	82.3	0.0874	0.032	0	7.224935	2.645285	0	2.6336
680	1.004472	78.3	0.0468	0.017	0	3.680827	1.337053	0	1.3311
690	1.005531	69.7	0.0227	0.0082	0	1.590941	0.574701	0	0.57154
700	1.005002	71.6	0.0114	0.0041	0	0.820323	0.295028	0	0.29356
710	1.005143	74.3	0.0058	0.0021	0	0.433156	0.156832	0	0.15603
720	1.005567	61.6	0.0029	0.001	0	0.179634	0.061943	0	0.0616
730	1.006344	69.9	0.0014	0.0005	0	0.098481	0.035172	0	0.03495
740	1.006521	75.1	0.0007	0.0002	0	0.052913	0.015118	0	0.01502
750	1.007229	63.6	0.0003	0.0001	0	0.019218	0.006406	0	0.00636
760	1.006168	46.4	0.0002	0.0001	0	0.009337	0.004669	0	0.00464
770	1.006486	66.8	0.0001	0	0	0.006723	0	0	0
780	1.006875	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   1010.73 1061.375 1154.814 1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   95.62139
Y = K SUM o*s*y =   100.4127
Z = K SUM o*s*z =   109.2526
Y,0.5=   10.02061

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.313218
Y =Y/(X + Y + Z) =   0.328913

Hunter L, a, b values

L = 10*Y,0.5 =   100.2061
a =17.5*(1.02X-Y)/Y,0.5 =   -5.02764
b = 7.0*(Y-0.847Z)/Y,0.5 =   5.501636

Lightness = L
Saturation = (a,2 + b,2),0.5 =   7.452863
Hue = tanb/a,-1 =   -47.6

Appendix 2.3b. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL sinapic acid (autoclaved 15 min)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.44461	50	0.0014	0	0.0065	0.031123	0	0.144498	0
390	0.569939	54.6	0.0042	0.0001	0.0201	0.130698	0.003112	0.625485	0.00546
400	0.626103	82.8	0.0143	0.0004	0.0679	0.741331	0.020737	3.520026	0.03312
410	0.648743	91.5	0.0435	0.0012	0.2074	2.582157	0.071232	12.31125	0.1098
420	0.663985	93.4	0.1344	0.004	0.6456	8.334981	0.248065	40.03768	0.3736
430	0.685991	86.7	0.2839	0.0116	1.3856	16.88508	0.689915	82.40919	1.00572
440	0.717975	104.9	0.3483	0.023	1.7471	26.23242	1.732258	131.5838	2.4127
450	0.754598	117	0.3362	0.038	1.7721	29.68243	3.354944	156.4552	4.446
460	0.791865	117.8	0.2908	0.06	1.6692	27.1263	5.596899	155.7057	7.068
470	0.826633	114.9	0.1954	0.091	1.2876	18.55911	8.643187	122.2963	10.4559
480	0.859538	115.9	0.0956	0.139	0.813	9.523713	13.84724	80.99141	16.1101
490	0.884569	108.8	0.032	0.208	0.4652	3.079714	20.01814	44.77134	22.6304
500	0.904939	109.4	0.0049	0.323	0.272	0.485101	31.97709	26.92808	35.3362
510	0.919328	107.8	0.0093	0.503	0.1582	0.921663	49.84908	15.67818	54.2234
520	0.928841	104.8	0.0633	0.71	0.0782	6.16178	69.11317	7.612183	74.408
530	0.936805	107.7	0.1655	0.862	0.0422	16.69794	86.97052	4.257722	92.8374
540	0.945169	104.4	0.2904	0.954	0.0203	28.65542	94.13659	2.003116	99.5976
550	0.951667	104	0.4334	0.995	0.0087	42.89506	98.47851	0.861068	103.48
560	0.958445	100	0.5945	0.995	0.0039	56.97957	95.36531	0.373794	99.5
570	0.96395	96.3	0.7621	0.952	0.0021	70.74452	88.37263	0.19494	91.6776
580	0.955923	95.8	0.9163	0.87	0.0017	83.9124	79.67237	0.155682	83.346
590	0.970918	88.7	1.0263	0.757	0.0011	88.3854	65.19317	0.094732	67.1459
600	0.976769	90	1.0622	0.631	0.0008	93.37716	55.47071	0.070327	56.79
610	0.981344	89.6	1.0026	0.503	0.0003	88.15704	44.228	0.026379	45.0688
620	0.985283	87.7	0.8544	0.381	0.0002	73.82808	32.92193	0.017282	33.4137
630	0.98653	83.8	0.6424	0.265	0	53.10797	21.90786	0	22.207
640	0.988472	83.7	0.4479	0.175	0	37.05707	14.47865	0	14.6475
650	0.989515	80	0.2835	0.107	0	22.4422	8.470248	0	8.56
660	0.987709	82.2	0.1649	0.061	0	13.38817	4.952569	0	5.0142
670	0.992893	82.3	0.0874	0.032	0	7.1419	2.614883	0	2.6336
680	0.99422	78.3	0.0468	0.017	0	3.643258	1.323406	0	1.3311
690	0.996038	69.7	0.0227	0.0082	0	1.575921	0.569275	0	0.57154
700	0.994883	71.6	0.0114	0.0041	0	0.812064	0.292058	0	0.29356
710	0.996003	74.3	0.0058	0.0021	0	0.429217	0.155406	0	0.15603
720	0.997193	61.6	0.0029	0.001	0	0.178139	0.061427	0	0.0616
730	0.999403	69.9	0.0014	0.0005	0	0.097802	0.034929	0	0.03495
740	0.998841	75.1	0.0007	0.0002	0	0.052509	0.015003	0	0.01502
750	1.000387	63.6	0.0003	0.0001	0	0.019087	0.006362	0	0.00636
760	0.999157	46.4	0.0002	0.0001	0	0.009272	0.004636	0	0.00464
770	0.999684	66.8	0.0001	0	0	0.006678	0	0	0
780	1.000387	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   934.0714   1000.862   889.1254   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   88.36901
Y = K SUM o*s*y =   94.68777
Z = K SUM o*s*z =   84.11683
Y,0.5=   9.730764

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y + Z) =   0.330755
Y =Y/(X + Y + Z) =   0.354405

Hunter L, a, b values
L = 10*Y,0.5 =   97.30764
a =17.5*(1.02X-Y)/Y,0.5 =   -8.18529
b = 7.0*(Y-0.847Z)/Y,0.5 =   16.86257

Lightness = L
Saturation = (a.2 + b.2),0.5 =   18.74421
Hue = tanb/a,-1 =   -64.1

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Appendix 2.4a. ^{1}H NMR spectrum of the unknown sample, identified
to be a mixture of sinapic acid and syringaldehyde.

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Appendix 2.4b. ^{13}C NMR spectrum of the unknown sample, identified to be a mixture of sinapic acid and syringaldehyde.

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Appendix 2.4c. ^{1}H NMR spectrum of standard syringaldehyde.

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Appendix 2.4d. ^{13}C NMR spectrum of standard syringaldehyde.
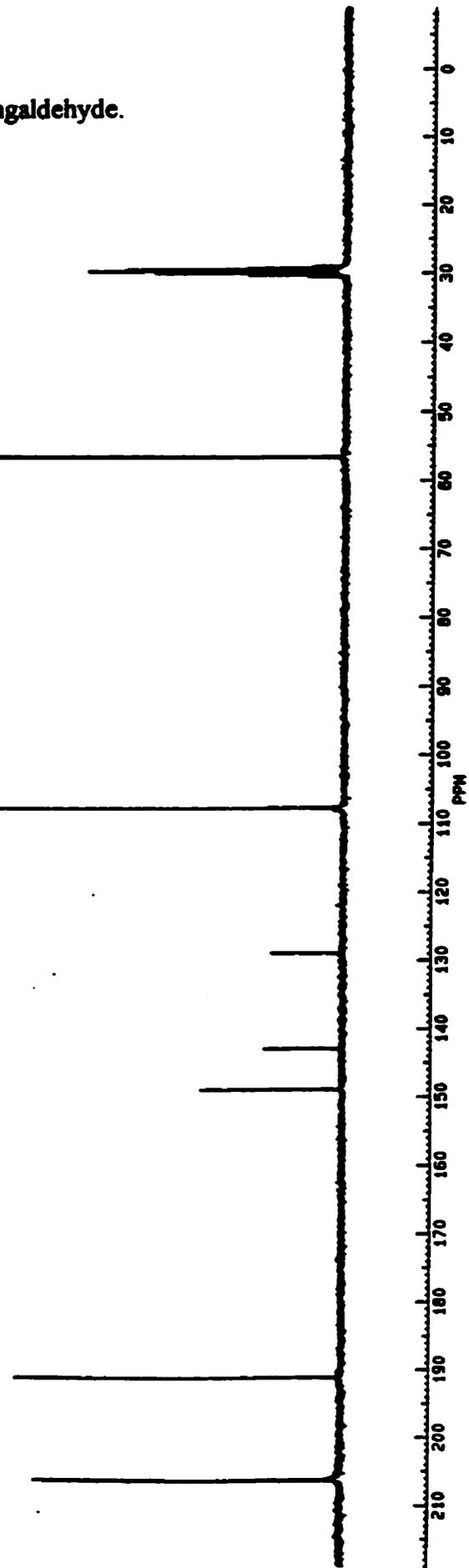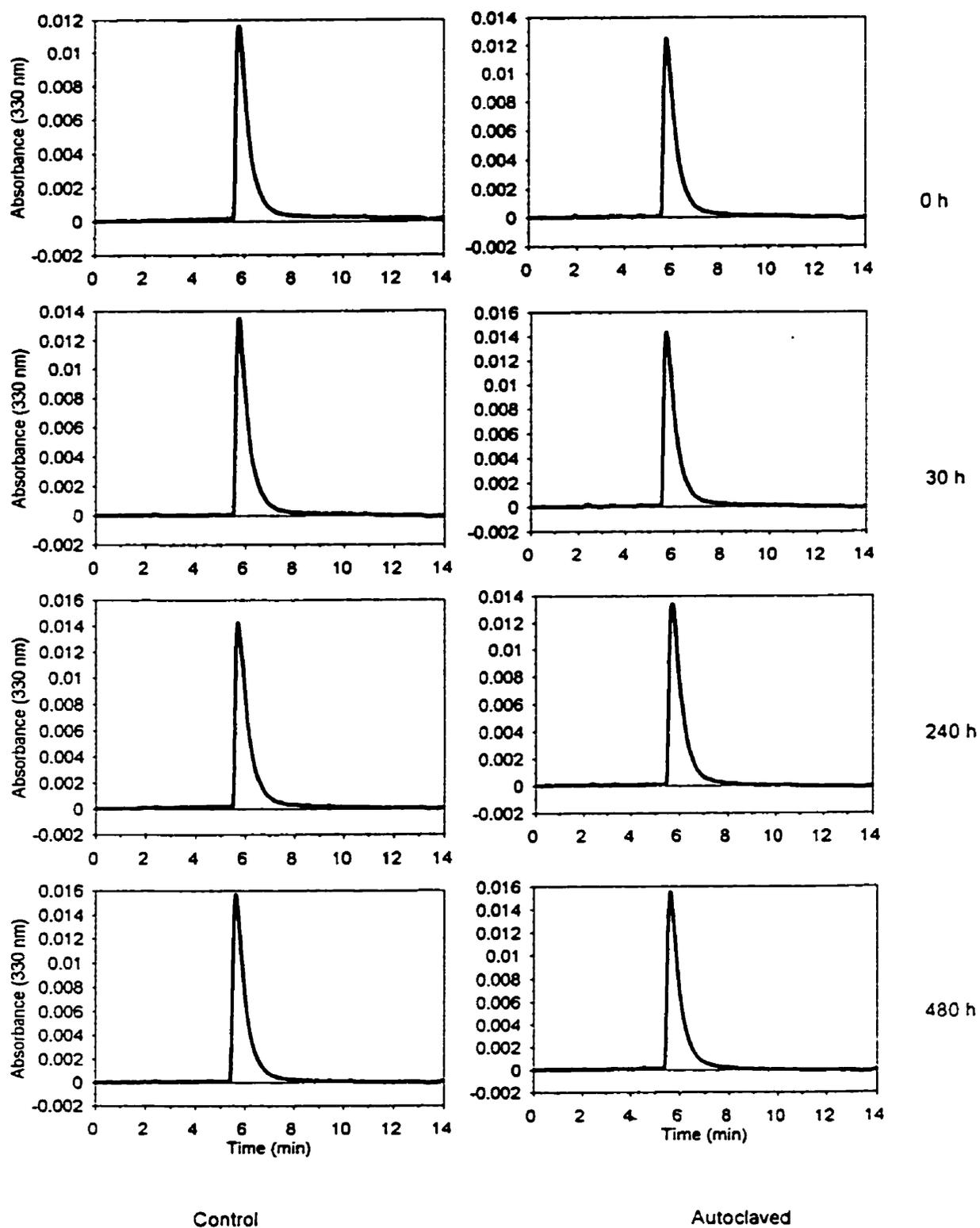
Appendix 2.5. The Effect of autoclaving on the structure of sinapine (shows no effect).

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Appendix 3.1. Concentration of sinapic acid, thomasidioic acid, 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5, 7-dimethoxy-2-naphthoic acid as a function of time during alkaline oxidation of 0.446 mmol/L sinapic acid solutions in phosphate-boric acid buffers of pH 7, 8.5 and 10 [a].

pH 7			pH 8.5			pH 10		
Time (h)	conc. (mmol/L)		Time (h)	conc. (mmol/L)		Time (h)	conc. (mmol/L)	
	SA [b]	TA		SA [b]	TA		SA [b]	TA
0	0.446	0	0	0.446	0	0	0.446	0
4.93	0.349	nd	0.82	0.351	0.014	0.45	0.374	0.026
9.93	0.316	0.017	2.62	0.310	0.033	2.09	0.284	0.069
16.35	0.259	0.037	5.01	0.251	0.076	4.48	0.216	0.113
22.65	0.225	0.060	7.43	0.229	0.077	5.36	0.176	0.140
27.45	0.198	0.066	10.71	0.179	0.088	7.10	0.113	0.165
34.30	0.142	0.074	16.01	0.092	0.112	10.38	0.045	0.174
50.68	0.085	0.090	20.00	0.063	0.145	15.00	0.030	0.180
54.68	0.090	0.094	24.33	0.045	0.168	20.02	0.012	0.189
132.58	0.007	0.143	26.00	0.041	0.171	24.01	0.007	0.196
169.33	0.001	0.184	169.02	nd	0.136	168.06	nd	0.041
240.10	nd	0.141	240.51	nd	0.078	240.92	nd	0.003

	BQ	NA		BQ	NA		BQ	NA
132.58	nd	nd	26.00	nd	nd	24.01	nd	nd
169.33	0.008	0.017	169.02	0.011	0.072	168.06	0.044	0.132
240.10	0.013	0.030	240.51	0.044	0.125	240.92	0.043	0.144

[a] SA = Sinapic Acid, TA = Thomasidioic Acid, BQ = 2,6-Dimethoxy-*p*-Benzoquinone, and NA = 6-Hydroxy-5, 7-Dimethoxy-2-Naphthoic Acid, nd = not detected.
[b] Reaction rate constant *k* value as in Table 3.1.

Appendix 3.2. Regression output for 1st order and 2nd order reaction fit of concentrations (mmol/L) vs reaction times (hour) of sinapic acid in phosphate-boric acid buffers of pH 7, 8.5 and 10.

pH 10 1st Order		pH 10 2nd Order	
Constant	0.0828	Constant	-14.4130
Std Err of Y Est	0.1649	Std Err of Y Est	19.0897
R Squared	0.9888	R Squared	0.8465
No. of Observations	10	No. of Observations	10
Degrees of Freedom	8	Degrees of Freedom	8
X Coefficient(s)	0.1753	X Coefficient(s)	5.0782
Std Err of Coef.	0.0066	Std Err of Coef.	0.7647

pH 8.5 1st Order		pH 8.5 2nd Order	
Constant	0.0751	Constant	0.1581
Std Err of Y Est	0.0813	Std Err of Y Est	2.3100
R Squared	0.9925	R Squared	0.9324
No. of Observations	10	No. of Observations	10
Degrees of Freedom	8	Degrees of Freedom	8
X Coefficient(s)	0.0905	X Coefficient(s)	0.8314
Std Err of Coef.	0.0028	Std Err of Coef.	0.0792

pH 7 1st Order		pH 7 2nd Order	
Constant	0.0375	Constant	-103.9770
Std Err of Y Est	0.0496	Std Err of Y Est	193.8545
R Squared	0.9796	R Squared	0.6457
No. of Observations	12	No. of Observations	16
Degrees of Freedom	10	Degrees of Freedom	14
X Coefficient(s)	0.0308	X Coefficient(s)	5.3097
Std Err of Coef.	0.0014	Std Err of Coef.	1.0512

Appendix 3.3. Effect of time on the Hunter L a b values of 0.446 mmol/L sinapic acid solutions during oxidations in phosphate-boric acid buffers of pH 7, 8.5 and 10 [a].

Time (h)	Hunter color value		
	L	a	b
	Control [b]		
	99.77±0.15	-5.55±0.31	5.47±0.25
	pH 7		
0	99.18±0.71^A	-5.22±0.17^B	5.85±0.48^D
18	96.84±0.54^B	-5.92±0.37^C	12.52±0.52^C
28	96.33±0.38^B	-4.47±0.29^A	15.53±0.06^B
168	88.68±0.91^C	-7.72±0.39^D	19.46±0.58^A
	pH 8.5		
0	98.40±0.53^A	-5.80±0.17^A	7.70±0.53^D
18	92.32±1.16^B	-6.46±0.36^A	14.23±0.57^B
28	91.00±2.38^{BC}	-8.27±1.45^B	12.84±1.16^C
169	88.40±1.28^C	-5.69±0.45^A	19.08±0.39^A
	pH 10		
0	99.43±0.81^A	-7.90±0.61^C	11.47±0.59^B
18	93.49±1.32^B	-6.49±0.36^B	12.49±0.50^B
28	97.00±1.10^C	-6.25±0.27^B	11.69±1.10^B
169	86.97±0.91^D	-2.42±0.33^A	17.9±0.98^A

[a] Mean of three replicates±SD. Column values with the same letters were not significantly different (p ≤ 0.05).

[b] Control = Sinapic acid in deionized water with a natural pH of 4.3 at zero time.

Appendix 3.4a. ANOVA of Appendix 3.3 (pH 7). The effect of air oxidation time on the color of a 100 μg/mL sinapic acid in phosphate-boric acid solution of pH 7.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	187.1008667	62.3669556	141.03	0.0001
Error	8	3.5378000	0.4422250		
Corrected Total	11	190.6386667			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	17.40222500	5.80074167	58.02	0.0001
Error	8	0.79986667	0.09998333		
Corrected Total	11	18.20209167			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	297.3850917	99.1283639	468.14	0.0001
Error	8	1.6940000	0.2117500		
Corrected Total	11	299.0790917			

```
Options linesize=78;
Title 'Sinapic acid in alkaline solution pH 7, Table 3-1-1';
Data;
  Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;*
0        98.8       -5.3      5.6
0        100.0      -5.4      6.4
0        98.8       -5.0      5.5
18       96.3       -5.6      12.1
18       96.8       -5.9      12.4
18       97.4       -6.3      13.1
27       96.2       -4.8      15.5
27       96.8       -4.5      15.5
27       96.0       -4.2      15.6
168      88.8       -7.8      19.3
168      89.5       -7.3      19.0
168      87.7       -8.1      20.1
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;
```

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Appendix 3.4b. ANOVA of Appendix 3.3 (pH 8.5). The effect of air oxidation time on the color of a 100 μg/mL sinapic acid in phosphate-boric acid solution of pH 8.5.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	161.6964000	53.8988000	24.18	0.0002
Error	8	17.8344000	2.2293000		
Corrected Total	11	179.5308000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	12.82282500	4.27427500	6.90	0.0131
Error	8	4.95406667	0.61925833		
Corrected Total	11	17.77689167			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	197.2083667	65.7361222	125.39	0.0001
Error	8	4.1941333	0.5242667		
Corrected Total	11	201.4025000			

Options linesize=78;
Title 'Sinapic acid in alkaline solution pH 8.5, Table 3-1-2';
Data;
  Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0   99.0   -5.9   8.1
0   98.2   -5.9   7.1
0   98.0   -5.6   7.9
18   91.1   -6.8   14.4
18   92.5   -6.1   13.6
18   93.4   -6.5   14.7
27   93.7   -6.9   14.1
27   89.2   -9.8   11.9
27   90.1   -8.1   12.5
168   89.5   -6.1   18.7
168   88.7   -5.8   19.0
168   87.0   -5.2   19.5
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;

Appendix 3.4c. ANOVA of Appendix 3.3 (pH 10). The effect of air oxidation time on the color of a 100 μg/mL sinapic acid in phosphate-boric acid solution of pH 10.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	264.2084667	88.0694889	79.68	0.0001
Error	8	8.8420000	1.1052500		
Corrected Total	11	273.0504667			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	49.92996667	16.64332222	97.89	0.0001
Error	8	1.36020000	0.17002500		
Corrected Total	11	51.29016667			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	84.41155833	28.13718611	40.70	0.0001
Error	8	5.53126667	0.69140833		
Corrected Total	11	89.94282500			

```
Options linesize=78;
Title 'Sinapic acid in alkaline solution pH 10, Table 3-1-3';
Data;
  Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0        98.5      -7.8      11.9
0        100       -7.4      10.8
0        99.8      -8.6      11.7
18       94.5      -6.1      12.0
18       93.9      -6.6      12.5
18       92.0      -6.8      13.0
27       95.9      -6.5      12.9
27       98.1      -6.2      11.1
27       97.0      -6.0      11.0
168      87.8      -2.8      17.9
168      87.1      -2.4      18.9
168      86.0      -2.1      17.0
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;
```

Appendix 3.5. Effect of time on the Hunter L a b values of sinapic acid solutions during oxidations in ammonium bicarbonate buffers of 8.5 and 10 [a].

Time (h)	Hunter color value		
	L	a	b
	pH 8.5		
0	97.60 ± 0.53^{A}	-7.92 ± 0.20^{D}	12.73 ± 0.38^{C}
1	91.92 ± 1.41^{B}	-5.10 ± 0.13^{B}	25.41 ± 2.23^{A}
3	92.90 ± 0.77^{B}	-7.29 ± 0.51^{C}	15.75 ± 0.42^{B}
72	88.09 ± 0.59^{C}	-4.49 ± 0.26^{A}	23.43 ± 0.47^{A}
	pH 10		
0	98.74 ± 0.31^{A}	-9.50 ± 0.47^{C}	14.70 ± 0.46^{BC}
1	94.53 ± 1.07^{B}	-6.52 ± 0.17^{B}	15.45 ± 0.62^{B}
3	96.17 ± 0.90^{C}	-5.99 ± 0.15^{AB}	14.09 ± 0.26^{C}
72	88.91 ± 0.82^{D}	-5.61 ± 0.27^{A}	22.96 ± 0.62^{A}

[a] Mean of three replicates$\pm$SD. Column values with the same letters were not significantly different ($p \leq 0.05$).

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Appendix 3.6a. ANOVA of Appendix 3.5 (pH 8.5). The effect of air oxidation time on the color of a 100 μg/mL sinapic acid in ammonium bicarbonate buffer of pH 8.5.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	137.5973667	45.8657889	56.85	0.0001
Error	8	6.4538000	0.8067250		
Corrected Total	11	144.0511667			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	24.71716667	8.23905556	86.64	0.0001
Error	8	0.76080000	0.09510000		
Corrected Total	11	25.47796667			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	330.2868917	110.0956306	80.16	0.0001
Error	8	10.9870000	1.3733750		
Corrected Total	11	341.2738917			

```
Options linesize=78;
Title 'Sinapic acid in alkaline solution pH 8.5 (NH4), Table 3-2-1';
Data;
  Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0        98.0     -8.1      13.0
0        97.0     -7.7      12.3
0        97.8     -7.9      12.9
1        93.0     -5.1      25.1
1        92.4     -5.2      27.8
1        90.3     -4.9      23.4
3        93.1     -7.6      16.2
3        92.1     -6.7      15.7
3        93.6     -7.6      15.4
72       88.0     -4.5      23.8
72       87.6     -4.8      23.6
72       88.7     -4.2      22.9
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;
```

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Appendix 3.6b. ANOVA of Appendix 3.5 (pH 10). The effect of air oxidation time on the color of a 100 μg/mL sinapic acid in ammonium bicarbonate buffer of pH 10.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	155.8399583	51.9466528	75.97	0.0001
Error	8	5.4705333	0.6838167		
Corrected Total	11	161.3104917			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	28.22620000	9.40873333	109.84	0.0001
Error	8	0.68526667	0.08565833		
Corrected Total	11	28.91146667			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	154.7710917	51.5903639	198.08	0.0001
Error	8	2.0836000	0.2604500		
Corrected Total	11	156.8546917			

```
Options linesize=78;
Title 'Sinapic acid in alkaline solution pH 10 (NH4), Table 3-2-2';
Data;
   Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0        98.8       -9.7      14.6
0        98.4       -9.8      14.3
0        99.0       -8.9      15.2
1        94.0       -6.7      15.9
1        93.8       -6.5      15.7
1        95.8       -6.4      14.7
3        96.3       -6.0      14.2
3        95.2       -6.1      14.3
3        97.0       -5.8      13.8
72       88.1       -5.4      23.1
72       89.7       -5.9      22.3
72       88.9       -5.5      23.5
Proc GLM;
   Classes Time;
   Model L a b = Time;
   Means Time / Duncan;
```

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Appendix 3.7a. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL sinapic acid (pH 7.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.869501	50	0.0014	0	0.0065	0.060865	0	0.282588	0
390	0.91257	54.6	0.0042	0.0001	0.0201	0.20927	0.004983	1.001509	0.00546
400	0.94361	82.8	0.0143	0.0004	0.0679	1.117272	0.031252	5.305088	0.03312
410	0.962529	91.5	0.0435	0.0012	0.2074	3.831104	0.105686	18.266	0.1098
420	0.97143	93.4	0.1344	0.004	0.6456	12.19432	0.362926	58.5763	0.3736
430	0.97502	86.7	0.2839	0.0116	1.3856	23.99928	0.980597	117.1307	1.00572
440	0.977215	104.9	0.3483	0.023	1.7471	35.70419	2.357727	179.095	2.4127
450	0.977937	117	0.3362	0.038	1.7721	38.46752	4.347906	202.7611	4.446
460	0.978211	117.8	0.2908	0.06	1.6692	33.50984	6.913998	192.3474	7.068
470	0.977593	114.9	0.1954	0.091	1.2876	21.94839	10.22161	144.6302	10.4559
480	0.976117	115.9	0.0956	0.139	0.813	10.81542	15.72534	91.97629	16.1101
490	0.97622	108.8	0.032	0.208	0.4652	3.398808	22.09225	49.41017	22.6304
500	0.977353	109.4	0.0049	0.323	0.272	0.52392	34.53592	29.08288	35.3362
510	0.974815	107.8	0.0093	0.503	0.1582	0.977291	52.85777	16.62445	54.2234
520	0.975226	104.8	0.0633	0.71	0.0782	6.469492	72.5646	7.992327	74.408
530	0.974438	107.7	0.1655	0.862	0.0422	17.36873	90.4643	4.428763	92.8374
540	0.975809	104.4	0.2904	0.954	0.0203	29.58433	97.18818	2.06805	99.5976
550	0.976495	104	0.4334	0.995	0.0087	44.01412	101.0477	0.883532	103.48
560	0.97526	100	0.5945	0.995	0.0039	57.97922	97.03839	0.380351	99.5
570	0.974438	96.3	0.7621	0.952	0.0021	71.51424	89.33415	0.197061	91.6776
580	0.982586	95.8	0.9163	0.87	0.0017	86.25292	81.89462	0.160024	83.346
590	0.97945	88.7	1.0263	0.757	0.0011	89.16204	65.76602	0.095565	67.1459
600	0.978315	90	1.0622	0.631	0.0008	93.52491	55.55848	0.070439	56.79
610	0.978005	89.6	1.0026	0.503	0.0003	87.85709	44.07752	0.026289	45.0688
620	0.980551	87.7	0.8544	0.381	0.0002	73.47357	32.76385	0.017199	33.4137
630	0.98231	83.8	0.6424	0.265	0	52.88081	21.81416	0	22.207
640	0.980655	83.7	0.4479	0.175	0	36.76399	14.36414	0	14.6475
650	0.98093	80	0.2835	0.107	0	22.2475	8.396763	0	8.56
660	0.985317	82.2	0.1649	0.061	0	13.35576	4.940577	0	5.0142
670	0.978865	82.3	0.0874	0.032	0	7.040993	2.577938	0	2.6336
680	0.980414	78.3	0.0468	0.017	0	3.592666	1.305028	0	1.3311
690	0.980724	69.7	0.0227	0.0082	0	1.551691	0.560523	0	0.57154
700	0.982552	71.6	0.0114	0.0041	0	0.801998	0.288438	0	0.29356
710	0.981689	74.3	0.0058	0.0021	0	0.423049	0.153173	0	0.15603
720	0.981999	61.6	0.0029	0.001	0	0.175424	0.060491	0	0.0616
730	0.980379	69.9	0.0014	0.0005	0	0.09594	0.034264	0	0.03495
740	0.980655	75.1	0.0007	0.0002	0	0.051553	0.014729	0	0.01502
750	0.979897	63.6	0.0003	0.0001	0	0.018696	0.006232	0	0.00636
760	0.982483	46.4	0.0002	0.0001	0	0.009117	0.004559	0	0.00464
770	0.981448	66.8	0.0001	0	0	0.006556	0	0	0
780	0.980655	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   982.9739   1032.757   1122.809   1057.013

CIE 1931 tristimulus values
X = K SUM o*s*x =   92.99548
Y = K SUM o*s*y =   97.70525
Z = K SUM o*s*z =   106.2248
Y,0.5=   9.884597

CIE 1931 (X, Y)-chromaticity coordinates
X =X/(X + Y +Z) =   0.313195
Y =Y/(X + Y + Z) =   0.329056

Hunter L, a, b values
L = 10*Y,0.5 =   98.84597
a =17.5°(1.02X-Y)/Y,0.5 =   -5.04548
b = 7.0°(Y-0.847Z)/Y,0.5 =   5.476195

Lightness = L
Saturation = (a.2 + b.2),0.5 =   7.446181
Hue = tanb/a,-1 =   -47.3

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Appendix 3.7b. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL sinapic acid (pH 8.5)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.128431	50	0.0014	0	0.0065	0.00899	0	0.04174	0
390	0.273303	54.6	0.0042	0.0001	0.0201	0.062674	0.001492	0.299939	0.00546
400	0.495371	82.8	0.0143	0.0004	0.0679	0.586539	0.016407	2.785034	0.03312
410	0.714778	91.5	0.0435	0.0012	0.2074	2.844997	0.078483	13.56442	0.1098
420	0.85827	93.4	0.1344	0.004	0.6456	10.77383	0.32065	51.75288	0.3736
430	0.928025	86.7	0.2839	0.0116	1.3856	22.84253	0.933333	111.4851	1.00572
440	0.958782	104.9	0.3483	0.023	1.7471	35.03071	2.313254	175.7168	2.4127
450	0.972147	117	0.3362	0.038	1.7721	38.23979	4.322165	201.5608	4.446
460	0.978452	117.8	0.2908	0.06	1.6692	33.51809	6.915699	192.3947	7.068
470	0.980793	114.9	0.1954	0.091	1.2876	22.02022	10.25507	145.1036	10.4559
480	0.979931	115.9	0.0956	0.139	0.813	10.85768	15.78679	92.33569	16.1101
490	0.980345	108.8	0.032	0.208	0.4652	3.413168	22.18559	49.61893	22.6304
500	0.981344	109.4	0.0049	0.323	0.272	0.526059	34.67697	29.20166	35.3362
510	0.978486	107.8	0.0093	0.503	0.1582	0.980972	53.05686	16.68707	54.2234
520	0.978555	104.8	0.0633	0.71	0.0782	6.491579	72.81234	8.019612	74.408
530	0.977284	107.7	0.1655	0.862	0.0422	17.41945	90.7285	4.441697	92.8374
540	0.978624	104.4	0.2904	0.954	0.0203	29.66969	97.4686	2.074017	99.5976
550	0.980104	104	0.4334	0.995	0.0087	44.17679	101.4211	0.886798	103.48
560	0.978968	100	0.5945	0.995	0.0039	58.19964	97.4073	0.381797	99.5
570	0.979209	96.3	0.7621	0.952	0.0021	71.86434	89.77149	0.198025	91.6776
580	0.99035	95.8	0.9163	0.87	0.0017	86.93442	82.54169	0.161288	83.346
590	0.984729	88.7	1.0263	0.757	0.0011	89.64264	66.12051	0.09608	67.1459
600	0.982966	90	1.0622	0.631	0.0008	93.96957	55.82263	0.070774	56.79
610	0.98231	89.6	1.0026	0.503	0.0003	88.24381	44.27153	0.026404	45.0688
620	0.985179	87.7	0.8544	0.381	0.0002	73.82031	32.91847	0.01728	33.4137
630	0.987015	83.8	0.6424	0.265	0	53.13409	21.91864	0	22.207
640	0.985075	83.7	0.4479	0.175	0	36.9297	14.42888	0	14.6475
650	0.985213	80	0.2835	0.107	0	22.34464	8.433426	0	8.56
660	0.990106	82.2	0.1649	0.061	0	13.42067	4.96459	0	5.0142
670	0.982241	82.3	0.0874	0.032	0	7.065278	2.586829	0	2.6336
680	0.98466	78.3	0.0468	0.017	0	3.608226	1.31068	0	1.3311
690	0.984245	69.7	0.0227	0.0082	0	1.557262	0.562535	0	0.57154
700	0.986287	71.6	0.0114	0.0041	0	0.805047	0.289534	0	0.29356
710	0.985006	74.3	0.0058	0.0021	0	0.424478	0.15369	0	0.15603
720	0.985733	61.6	0.0029	0.001	0	0.176091	0.060721	0	0.0616
730	0.983346	69.9	0.0014	0.0005	0	0.09623	0.034368	0	0.03495
740	0.984037	75.1	0.0007	0.0002	0	0.051731	0.01478	0	0.01502
750	0.982655	63.6	0.0003	0.0001	0	0.018749	0.00625	0	0.00636
760	0.985975	46.4	0.0002	0.0001	0	0.00915	0.004575	0	0.00464
770	0.984902	66.8	0.0001	0	0	0.006579	0	0	0
780	0.983657	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   981.7864   1036.916   1098.922   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   92.88314
Y = K SUM o*s*y =   98.09878
Z = K SUM o*s*z =   103.9649
Y,0.5=   9.904483

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.314915
Y =Y/(X + Y + Z) =   0.332598

Hunter L, a, b values

L = 10*Y,0.5 =   99.04483
a =17.5*(1.02X-Y)/Y,0.5 =   -5.93314
b = 7.0*(Y-0.847Z)/Y,0.5 =   7.096138

Lightness = L
Saturation = (a,2 + b,2),0.5 =   9.249721
Hue = tanb/a,-1 =   -50.1

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Appendix 3.7c. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-
100 ug/mL sinapic acid (pH 10.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.001094	50	0.0014	0	0.0065	7.66E-05	0	0.000355	0
390	0.001799	54.6	0.0042	0.0001	0.0201	0.000412	9.82E-06	0.001974	0.00546
400	0.009641	82.8	0.0143	0.0004	0.0679	0.011415	0.000319	0.054202	0.03312
410	0.115053	91.5	0.0435	0.0012	0.2074	0.457938	0.012633	2.183363	0.1098
420	0.442428	93.4	0.1344	0.004	0.6456	5.553782	0.165291	26.67799	0.3736
430	0.760213	86.7	0.2839	0.0116	1.3856	18.71199	0.764562	91.32557	1.00572
440	0.914238	104.9	0.3483	0.023	1.7471	33.40323	2.205783	167.5532	2.4127
450	0.968737	117	0.3362	0.038	1.7721	38.10567	4.307006	200.8538	4.446
460	0.986079	117.8	0.2908	0.06	1.6892	33.77936	6.969606	193.8944	7.068
470	0.991081	114.9	0.1954	0.091	1.2876	22.25121	10.36264	146.6257	10.4559
480	0.995933	115.9	0.0956	0.139	0.813	11.03497	16.04458	93.84345	16.1101
490	0.996458	108.8	0.032	0.208	0.4652	3.469267	22.55024	50.43447	22.6304
500	0.995303	109.4	0.0049	0.323	0.272	0.533542	35.17023	29.61703	35.3362
510	0.995618	107.8	0.0093	0.503	0.1582	0.998147	53.98578	16.97923	54.2234
520	0.993033	104.8	0.0633	0.71	0.0782	6.587619	73.88957	8.13826	74.408
530	0.992823	107.7	0.1655	0.862	0.0422	17.69643	92.17113	4.512322	92.8374
540	0.995058	104.4	0.2904	0.954	0.0203	30.16794	99.10541	2.108847	99.5976
550	0.996668	104	0.4334	0.995	0.0087	44.9234	103.1352	0.901785	103.48
560	1.001371	100	0.5945	0.995	0.0039	59.53151	99.63641	0.390535	99.5
570	1.007406	96.3	0.7621	0.952	0.0021	73.93376	92.35656	0.203728	91.6776
580	0.995128	95.8	0.9163	0.87	0.0017	87.35389	82.93995	0.162067	83.346
590	1.002005	88.7	1.0263	0.757	0.0011	91.21533	67.28053	0.097766	67.1459
600	1.005849	90	1.0622	0.631	0.0008	96.15715	57.12216	0.072421	56.79
610	1.006981	89.6	1.0026	0.503	0.0003	90.46008	45.38343	0.027068	45.0688
620	1.004684	87.7	0.8544	0.381	0.0002	75.28186	33.57021	0.017622	33.4137
630	1.002181	83.8	0.6424	0.265	0	53.95053	22.25543	0	22.207
640	1.003132	83.7	0.4479	0.175	0	37.60665	14.69338	0	14.6475
650	1.002005	80	0.2835	0.107	0	22.72547	8.577163	0	8.56
660	0.995373	82.2	0.1649	0.061	0	13.49206	4.990999	0	5.0142
670	1.005213	82.3	0.0874	0.032	0	7.230517	2.647329	0	2.6336
680	1.00486	78.3	0.0468	0.017	0	3.682249	1.337569	0	1.3311
690	1.006203	69.7	0.0227	0.0082	0	1.592004	0.575085	0	0.57154
700	1.003625	71.6	0.0114	0.0041	0	0.819199	0.294624	0	0.29356
710	1.005284	74.3	0.0058	0.0021	0	0.433217	0.156854	0	0.15603
720	1.004754	61.6	0.0029	0.001	0	0.179489	0.061893	0	0.0616
730	1.008185	69.9	0.0014	0.0005	0	0.098661	0.035236	0	0.03495
740	1.007476	75.1	0.0007	0.0002	0	0.052963	0.015132	0	0.01502
750	1.010348	63.6	0.0003	0.0001	0	0.019277	0.006426	0	0.00636
760	1.006521	46.4	0.0002	0.0001	0	0.009341	0.00467	0	0.00464
770	1.009354	66.8	0.0001	0	0	0.006742	0	0	0
780	1.011271	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   983.5183   1054.781   1036.677   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   93.04699
Y = K SUM o*s*y =   99.78889
Z = K SUM o*s*z =   98.07615
Y,0.5=   9.989439

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.319846
Y =Y/(X + Y + Z) =   0.343021

Hunter L, a, b values

L = 10*Y,0.5 =   99.89439
a =17.5*(1.02X-Y)/Y,0.5 =   -8.5507
b = 7.0*(Y-0.847Z)/Y,0.5 =   11.71524

Lightness = L
Saturation = (a,2 + b,2),0.5 =   14.50384
Hue = tanb/a,-1 =   -53.9

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Appendix 3.7d. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL thomasidioic acid (pH 7.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.913949	50	0.0014	0	0.0065	0.063976	0	0.297034	0
390	0.948863	54.6	0.0042	0.0001	0.0201	0.217593	0.005181	1.041339	0.00546
400	0.965916	82.8	0.0143	0.0004	0.0679	1.143684	0.031991	5.430496	0.03312
410	0.976151	91.5	0.0435	0.0012	0.2074	3.885327	0.107181	18.52452	0.1098
420	0.982586	93.4	0.1344	0.004	0.6456	12.33436	0.367094	59.249	0.3736
430	0.986703	86.7	0.2839	0.0116	1.3856	24.28683	0.992347	118.5341	1.00572
440	0.988195	104.9	0.3483	0.023	1.7471	36.10534	2.384217	181.1072	2.4127
450	0.989828	117	0.3362	0.038	1.7721	38.93528	4.400775	205.2267	4.446
460	0.991081	117.8	0.2908	0.06	1.6692	33.95069	7.004958	194.8779	7.068
470	0.991673	114.9	0.1954	0.091	1.2876	22.2645	10.36883	146.7133	10.4559
480	0.995653	115.9	0.0956	0.139	0.813	11.03187	16.04007	93.81708	16.1101
490	0.997123	108.8	0.032	0.208	0.4652	3.471584	22.56529	50.46815	22.6304
500	0.996528	109.4	0.0049	0.323	0.272	0.534199	35.2135	29.85348	35.3362
510	0.997789	107.8	0.0093	0.503	0.1582	1.000323	54.10351	17.01625	54.2234
520	0.996738	104.8	0.0633	0.71	0.0782	6.612199	74.16527	8.168625	74.408
530	0.997649	107.7	0.1655	0.862	0.0422	17.78244	92.61911	4.534253	92.8374
540	1.000387	104.4	0.2904	0.954	0.0203	30.32949	99.63614	2.12014	99.5976
550	1.001618	104	0.4334	0.995	0.0087	45.14653	103.6474	0.906264	103.48
560	1.005708	100	0.5945	0.995	0.0039	59.78934	100.0679	0.392226	99.5
570	1.012907	96.3	0.7621	0.952	0.0021	74.33748	92.86088	0.20484	91.6776
580	1.007937	95.8	0.9163	0.87	0.0017	88.47826	84.00752	0.164153	83.346
590	1.008433	88.7	1.0263	0.757	0.0011	91.80049	67.71214	0.098393	67.1459
600	1.012089	90	1.0622	0.631	0.0008	96.75368	57.47653	0.07287	56.79
610	1.01312	89.6	1.0026	0.503	0.0003	91.01157	45.6601	0.027233	45.0688
620	1.010703	87.7	0.8544	0.381	0.0002	75.73287	33.77133	0.017728	33.4137
630	1.007972	83.8	0.6424	0.265	0	54.26228	22.38403	0	22.207
640	1.009354	83.7	0.4479	0.175	0	37.8399	14.78451	0	14.6475
650	1.008929	80	0.2835	0.107	0	22.88251	8.636432	0	8.56
660	1.00419	82.2	0.1649	0.061	0	13.61157	5.035209	0	5.0142
670	1.012124	82.3	0.0874	0.032	0	7.280228	2.66553	0	2.6336
680	1.011769	78.3	0.0468	0.017	0	3.707567	1.346766	0	1.3311
690	1.013334	69.7	0.0227	0.0082	0	1.603287	0.579161	0	0.57154
700	1.011413	71.6	0.0114	0.0041	0	0.825556	0.29691	0	0.29356
710	1.013263	74.3	0.0058	0.0021	0	0.436656	0.158099	0	0.15603
720	1.012978	61.6	0.0029	0.001	0	0.180958	0.062399	0	0.0616
730	1.015865	69.9	0.0014	0.0005	0	0.099413	0.035504	0	0.03495
740	1.015044	75.1	0.0007	0.0002	0	0.053361	0.015246	0	0.01502
750	1.018259	63.6	0.0003	0.0001	0	0.019428	0.006476	0	0.00636
760	1.014937	46.4	0.0002	0.0001	0	0.009419	0.004709	0	0.00464
770	1.017401	66.8	0.0001	0	0	0.006796	0	0	0
780	1.019011	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   1009.819   1061.22   1138.663   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   95.53519

Y = K SUM o*s*y =   100.3981

Z = K SUM o*s*z =   107.7247

Y,0.5=   10.01988

Hunter L, a, b values

L = 10*Y,0.5 =   100.1988

a =17.5*(1.02X-Y)/Y,0.5 =   -5.1561

b = 7.0*(Y-0.847Z)/Y,0.5 =   6.395985

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.314614

Y =Y/(X + Y + Z) =   0.330629

Lightness = L

Saturation = (a,2 + b,2),0.5 =   8.21547

Hue = tanb/a,-1 =   -51.1

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Appendix 3.7e. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-
100 ug/mL thomasidioic acid (pH 8.5)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.543881	50	0.0014	0	0.0065	0.038072	0	0.176761	0
390	0.67775	54.6	0.0042	0.0001	0.0201	0.155422	0.003701	0.743804	0.00546
400	0.806012	82.8	0.0143	0.0004	0.0679	0.95435	0.026695	4.531494	0.03312
410	0.898192	91.5	0.0435	0.0012	0.2074	3.575028	0.098621	17.04508	0.1098
420	0.948396	93.4	0.1344	0.004	0.6456	11.90518	0.354321	57.18737	0.3736
430	0.970816	86.7	0.2839	0.0116	1.3856	23.89579	0.976369	116.6256	1.00572
440	0.97969	104.9	0.3483	0.023	1.7471	35.79462	2.363699	179.5486	2.4127
450	0.984452	117	0.3362	0.038	1.7721	38.72381	4.376874	204.112	4.446
460	0.987605	117.8	0.2908	0.06	1.6692	33.83162	6.980389	194.1944	7.068
470	0.989515	114.9	0.1954	0.091	1.2876	22.21605	10.34627	146.394	10.4559
480	0.994429	115.9	0.0956	0.139	0.813	11.01831	16.02035	93.70177	16.1101
490	0.996283	108.8	0.032	0.208	0.4652	3.468658	22.54628	50.42561	22.6304
500	0.996248	109.4	0.0049	0.323	0.272	0.534048	35.2036	29.64514	35.3362
510	0.997754	107.8	0.0093	0.503	0.1582	1.000288	54.1016	17.01565	54.2234
520	0.996598	104.8	0.0633	0.71	0.0782	6.61127	74.15485	8.167478	74.408
530	0.997403	107.7	0.1655	0.862	0.0422	17.77807	92.59634	4.533139	92.8374
540	1.000141	104.4	0.2904	0.954	0.0203	30.32203	99.61164	2.119619	99.5976
550	1.001442	104	0.4334	0.995	0.0087	45.1386	103.6292	0.906105	103.48
560	1.005673	100	0.5945	0.995	0.0039	59.78726	100.0645	0.392212	99.5
570	1.012586	96.3	0.7621	0.952	0.0021	74.31392	92.83145	0.204775	91.6776
580	1.005037	95.8	0.9163	0.87	0.0017	88.2237	83.76581	0.16368	83.346
590	1.007724	88.7	1.0263	0.757	0.0011	91.73595	67.66453	0.098324	67.1459
600	1.011555	90	1.0622	0.631	0.0008	96.70263	57.44621	0.072832	56.79
610	1.012907	89.6	1.0026	0.503	0.0003	90.99243	45.6505	0.027227	45.0688
620	1.010667	87.7	0.8544	0.381	0.0002	75.73017	33.77012	0.017727	33.4137
630	1.008468	83.8	0.6424	0.265	0	54.28898	22.39505	0	22.207
640	1.009993	83.7	0.4479	0.175	0	37.86386	14.79387	0	14.6475
650	1.009815	80	0.2835	0.107	0	22.9026	8.644016	0	8.56
660	1.004613	82.2	0.1649	0.061	0	13.61731	5.037331	0	5.0142
670	1.01312	82.3	0.0874	0.032	0	7.287392	2.668153	0	2.6336
680	1.0128	78.3	0.0468	0.017	0	3.711345	1.348138	0	1.3311
690	1.014296	69.7	0.0227	0.0082	0	1.604809	0.579711	0	0.57154
700	1.012764	71.6	0.0114	0.0041	0	0.826658	0.297307	0	0.29356
710	1.014688	74.3	0.0058	0.0021	0	0.43727	0.158322	0	0.15603
720	1.014545	61.6	0.0029	0.001	0	0.181238	0.062496	0	0.0616
730	1.01758	69.9	0.0014	0.0005	0	0.09958	0.035564	0	0.03495
740	1.016972	75.1	0.0007	0.0002	0	0.053462	0.015275	0	0.01502
750	1.019978	63.6	0.0003	0.0001	0	0.019461	0.006487	0	0.00636
760	1.016936	46.4	0.0002	0.0001	0	0.009437	0.004719	0	0.00464
770	1.019512	66.8	0.0001	0	0	0.00681	0	0	0
780	1.02091	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   1007.357   1060.63   1128.05   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   95.30233
Y = K SUM o*s*y =   100.3423
Z = K SUM o*s*z =   106.7206
Y,0.5=   10.0171

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.315189
Y =Y/(X + Y + Z) =   0.331858

Hunter L, a, b values
L = 10*Y,0.5 =   100.171
a =17.5*(1.02X-Y)/Y,0.5 =   -5.47496
b = 7.0*(Y-0.847Z)/Y,0.5 =   6.953035

Lightness = L
Saturation = (a,2 + b,2),0.5 =   8.849855
Hue = tanb/a,-1 =   -51.8

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Appendix 3.7f. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL thomasidioic acid (pH 10.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.008278	50	0.0014	0	0.0065	0.000579	0	0.00269	0
390	0.043394	54.6	0.0042	0.0001	0.0201	0.009951	0.000237	0.047624	0.00546
400	0.187668	82.8	0.0143	0.0004	0.0679	0.222206	0.006216	1.055092	0.03312
410	0.475353	91.5	0.0435	0.0012	0.2074	1.892026	0.052194	9.02083	0.1098
420	0.744172	93.4	0.1344	0.004	0.6456	9.341563	0.278023	44.87287	0.3736
430	0.885813	86.7	0.2839	0.0116	1.3856	21.80351	0.89068	106.414	1.00572
440	0.940631	104.9	0.3483	0.023	1.7471	34.36752	2.26946	172.3902	2.4127
450	0.960333	117	0.3362	0.038	1.7721	37.77509	4.269642	199.1114	4.446
460	0.968976	117.8	0.2908	0.06	1.6692	33.19347	6.848721	190.5314	7.068
470	0.973378	114.9	0.1954	0.091	1.2876	21.85375	10.17754	144.0066	10.4559
480	0.975432	115.9	0.0956	0.139	0.813	10.80782	15.7143	91.91171	16.1101
490	0.978177	108.8	0.032	0.208	0.4652	3.405622	22.13654	49.50923	22.6304
500	0.980621	109.4	0.0049	0.323	0.272	0.525671	34.6514	29.18013	35.3382
510	0.979346	107.8	0.0093	0.503	0.1582	0.981834	53.10349	16.70173	54.2234
520	0.980517	104.8	0.0633	0.71	0.0782	6.504594	72.95832	8.035691	74.408
530	0.980345	107.7	0.1655	0.862	0.0422	17.47401	91.01266	4.455608	92.8374
540	0.982276	104.4	0.2904	0.954	0.0203	29.7804	97.8323	2.061757	99.5976
550	0.983519	104	0.4334	0.995	0.0087	44.33074	101.7745	0.889888	103.48
560	0.982552	100	0.5945	0.995	0.0039	58.4127	97.7639	0.383195	99.5
570	0.983001	96.3	0.7621	0.952	0.0021	72.14264	90.11914	0.198792	91.6776
580	0.995548	95.8	0.9163	0.87	0.0017	87.39074	82.97495	0.162135	83.346
590	0.987883	88.7	1.0263	0.757	0.0011	89.92972	66.33226	0.096388	67.1459
600	0.986357	90	1.0622	0.631	0.0008	94.29371	56.01519	0.071018	56.79
610	0.986253	89.6	1.0026	0.503	0.0003	88.598	44.44922	0.02651	45.0688
620	0.989515	87.7	0.8544	0.381	0.0002	74.14525	33.06336	0.017356	33.4137
630	0.992196	83.8	0.6424	0.265	0	53.413	22.03369	0	22.207
640	0.99136	83.7	0.4479	0.175	0	37.16531	14.52094	0	14.6475
650	0.993103	80	0.2835	0.107	0	22.52357	8.500958	0	8.56
660	0.998736	82.2	0.1649	0.061	0	13.53765	5.007863	0	5.0142
670	0.991778	82.3	0.0874	0.032	0	7.133876	2.611946	0	2.6336
680	0.994255	78.3	0.0468	0.017	0	3.643387	1.323452	0	1.3311
690	0.995059	69.7	0.0227	0.0082	0	1.574372	0.568716	0	0.57154
700	0.997824	71.6	0.0114	0.0041	0	0.814464	0.292921	0	0.29356
710	0.996738	74.3	0.0058	0.0021	0	0.429534	0.155521	0	0.15603
720	0.998456	61.6	0.0029	0.001	0	0.178364	0.061505	0	0.0616
730	0.996458	69.9	0.0014	0.0005	0	0.097513	0.034826	0	0.03495
740	0.997509	75.1	0.0007	0.0002	0	0.052439	0.014983	0	0.01502
750	0.996598	63.6	0.0003	0.0001	0	0.019015	0.006338	0	0.00636
760	1.000176	46.4	0.0002	0.0001	0	0.009282	0.004641	0	0.00464
770	0.999684	66.8	0.0001	0	0	0.006678	0	0	0
780	0.998491	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   979.7816   1039.833   1071.174   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   92.69347
Y = K SUM o*s*y =   98.37469
Z = K SUM o*s*z =   101.3397
Y,0.5=   9.918402

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.317001
Y =Y/(X + Y + Z) =   0.33643

Hunter L, a, b values
L = 10*Y,0.5 =   99.18402
a =17.5*(1.02X-Y)/Y,0.5 =   -6.75297
b = 7.0*(Y-0.847Z)/Y,0.5 =   8.850164

Lightness = L
Saturation = (a,2 + b,2),0.5 =   11.1323
Hue = tanb/a,-1 =   -52.7

Appendix 3.7g. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL 2-naphthoic acid (pH 7.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.900309	50	0.0014	0	0.0065	0.063022	0	0.2926	0
390	0.927797	54.6	0.0042	0.0001	0.0201	0.212762	0.005066	1.01822	0.00546
400	0.942318	82.8	0.0143	0.0004	0.0679	1.115742	0.03121	5.297823	0.03312
410	0.949296	91.5	0.0435	0.0012	0.2074	3.778436	0.104233	18.01489	0.1098
420	0.952872	93.4	0.1344	0.004	0.6456	11.96136	0.355693	57.45724	0.3736
430	0.954916	86.7	0.2839	0.0116	1.3856	23.50443	0.960378	114.7155	1.00572
440	0.957267	104.9	0.3483	0.023	1.7471	34.97536	2.309599	175.4392	2.4127
450	0.959658	117	0.3362	0.038	1.7721	37.74855	4.266641	198.9714	4.446
460	0.961076	117.8	0.2908	0.06	1.6692	32.92284	6.792882	188.978	7.068
470	0.96138	114.9	0.1954	0.091	1.2876	21.58437	10.05209	142.2315	10.4559
480	0.962562	115.9	0.0956	0.139	0.813	10.66523	15.50698	90.69908	16.1101
490	0.964153	108.8	0.032	0.208	0.4652	3.356796	21.81917	48.79942	22.6304
500	0.964628	109.4	0.0049	0.323	0.272	0.517098	34.08627	28.70423	35.3362
510	0.964967	107.8	0.0093	0.503	0.1582	0.967418	52.32378	16.4565	54.2234
520	0.966018	104.8	0.0633	0.71	0.0782	6.40841	71.87948	7.916867	74.408
530	0.968023	107.7	0.1655	0.862	0.0422	17.25438	89.86872	4.399606	92.8374
540	0.971737	104.4	0.2904	0.954	0.0203	29.46089	96.78268	2.059422	99.5976
550	0.974952	104	0.4334	0.995	0.0087	43.94459	100.888	0.882136	103.48
560	0.97773	100	0.5945	0.995	0.0039	58.12607	97.28417	0.381315	99.5
570	0.98345	96.3	0.7621	0.952	0.0021	72.17559	90.1603	0.198883	91.6776
580	0.991185	95.8	0.9163	0.87	0.0017	87.00775	82.61131	0.161424	83.346
590	0.987848	88.7	1.0263	0.757	0.0011	89.92654	66.32992	0.096384	67.1459
600	0.98948	90	1.0622	0.631	0.0008	94.59232	56.19257	0.071243	56.79
610	0.990698	89.6	1.0026	0.503	0.0003	88.99731	44.64956	0.02663	45.0688
620	0.990733	87.7	0.8544	0.381	0.0002	74.23647	33.10404	0.017377	33.4137
630	0.991255	83.8	0.6424	0.265	0	53.36234	22.0128	0	22.207
640	0.991046	83.7	0.4479	0.175	0	37.15354	14.51634	0	14.6475
650	0.99223	80	0.2835	0.107	0	22.50379	8.493492	0	8.56
660	0.9938	82.2	0.1649	0.061	0	13.47075	4.983114	0	5.0142
670	0.992579	82.3	0.0874	0.032	0	7.139641	2.614056	0	2.6336
680	0.993207	78.3	0.0468	0.017	0	3.639547	1.322058	0	1.3311
690	0.994045	69.7	0.0227	0.0082	0	1.572768	0.568136	0	0.57154
700	0.994639	71.6	0.0114	0.0041	0	0.811864	0.291986	0	0.29356
710	0.99415	74.3	0.0058	0.0021	0	0.428419	0.155117	0	0.15603
720	0.994779	61.6	0.0029	0.001	0	0.177707	0.061278	0	0.0616
730	0.995058	69.9	0.0014	0.0005	0	0.097376	0.034777	0	0.03495
740	0.995478	75.1	0.0007	0.0002	0	0.052332	0.014952	0	0.01502
750	0.995548	63.6	0.0003	0.0001	0	0.018995	0.006332	0	0.00636
760	0.996248	46.4	0.0002	0.0001	0	0.009245	0.004623	0	0.00464
770	0.996283	66.8	0.0001	0	0	0.006655	0	0	0
780	0.996248	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   985.9487   1033.444   1103.287   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   93.27692
Y = K SUM o*s*y =   97.77028
Z = K SUM o*s*z =   104.3778
Y,0.5=   9.887886

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.315738
Y =Y/(X + Y + Z) =   0.330948

Hunter L, a, b values

L = 10*Y,0.5 =   98.87886
a =17.5*(1.02X-Y)/Y,0.5 =   -4.65084
b = 7.0*(Y-0.847Z)/Y,0.5 =   6.62788

Lightness = L
Saturation = (a,2 + b,2),0.5 =   8.096857
Hue = tanb/a,-1 =   -54.8

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Appendix 3.7h. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-
100 ug/mL 2-naphthoic acid (pH 8.5)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.647331	50	0.0014	0	0.0065	0.045313	0	0.210383	0
390	0.824254	54.6	0.0042	0.0001	0.0201	0.189018	0.0045	0.904586	0.00546
400	0.884475	82.8	0.0143	0.0004	0.0679	1.047254	0.029294	4.972626	0.03312
410	0.903477	91.5	0.0435	0.0012	0.2074	3.596066	0.099202	17.14538	0.1098
420	0.910424	93.4	0.1344	0.004	0.6456	11.42652	0.340134	54.89769	0.3736
430	0.914881	86.7	0.2839	0.0116	1.3856	22.519	0.920114	109.9061	1.00572
440	0.920621	104.9	0.3483	0.023	1.7471	33.83641	2.221182	168.7229	2.4127
450	0.92529	117	0.3362	0.038	1.7721	36.39666	4.113841	191.8457	4.446
460	0.928351	117.8	0.2908	0.06	1.6692	31.80182	6.561587	182.5434	7.068
470	0.930082	114.9	0.1954	0.091	1.2876	20.88169	9.72484	137.6011	10.4559
480	0.928482	115.9	0.0956	0.139	0.813	10.28762	14.95793	87.48778	16.1101
490	0.931095	108.8	0.032	0.208	0.4652	3.241701	21.07106	47.12623	22.6304
500	0.934143	109.4	0.0049	0.323	0.272	0.500756	33.00905	27.79709	35.3362
510	0.932437	107.8	0.0093	0.503	0.1582	0.934806	50.55993	15.90175	54.2234
520	0.936245	104.8	0.0633	0.71	0.0782	6.210902	69.66415	7.672868	74.408
530	0.937595	107.7	0.1655	0.862	0.0422	16.71202	87.04389	4.261313	92.8374
540	0.940631	104.4	0.2904	0.954	0.0203	28.51782	93.68456	1.993497	99.5976
550	0.943643	104	0.4334	0.995	0.0087	42.53338	97.64817	0.853808	103.48
560	0.941755	100	0.5945	0.995	0.0039	55.98734	93.70463	0.367284	99.5
570	0.941226	96.3	0.7621	0.952	0.0021	69.07677	86.28931	0.190344	91.6776
580	0.964933	95.8	0.9163	0.87	0.0017	84.70328	80.42328	0.157149	83.346
590	0.954782	88.7	1.0263	0.757	0.0011	86.91647	64.10968	0.093158	67.1459
600	0.952169	90	1.0622	0.631	0.0008	91.02543	54.07367	0.068556	56.79
610	0.952069	89.6	1.0026	0.503	0.0003	85.52713	42.90858	0.025592	45.0688
620	0.9572	87.7	0.8544	0.381	0.0002	71.72385	31.9836	0.016789	33.4137
630	0.961447	83.8	0.6424	0.265	0	51.7577	21.35086	0	22.207
640	0.958513	83.7	0.4479	0.175	0	35.9339	14.03981	0	14.6475
650	0.960367	80	0.2835	0.107	0	21.78112	8.220739	0	8.56
660	0.971259	82.2	0.1649	0.061	0	13.16521	4.870088	0	5.0142
670	0.955822	82.3	0.0874	0.032	0	6.87525	2.517254	0	2.6336
680	0.958715	78.3	0.0468	0.017	0	3.513153	1.276145	0	1.3311
690	0.958445	69.7	0.0227	0.0082	0	1.516443	0.54779	0	0.57154
700	0.962867	71.6	0.0114	0.0041	0	0.78593	0.282659	0	0.29356
710	0.960198	74.3	0.0058	0.0021	0	0.413788	0.14982	0	0.15603
720	0.961616	61.6	0.0029	0.001	0	0.171783	0.059236	0	0.0616
730	0.957469	69.9	0.0014	0.0005	0	0.093698	0.033464	0	0.03495
740	0.959557	75.1	0.0007	0.0002	0	0.050444	0.014413	0	0.01502
750	0.956057	63.6	0.0003	0.0001	0	0.018242	0.006081	0	0.00636
760	0.962427	46.4	0.0002	0.0001	0	0.008931	0.004466	0	0.00464
770	0.959389	66.8	0.0001	0	0	0.006409	0	0	0
780	0.957167	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   951.533   998.519   1062.763   1057.013

CIE 1931 tristimulus values   CIE 1931 (X, Y)-chromaticity coordinates
X = K SUM o*s*x =   90.02098   X =X/(X + Y +Z) =   0.315829
Y = K SUM o*s*y =   94.46615   Y =Y/(X + Y + Z) =   0.331424
Z = K SUM o*s*z =   100.544
Y,0.5=   9.71937

Hunter L, a, b values
L = 10*Y,0.5 =   97.1937   Lightness = L
a =17.5*(1.02X-Y)/Y,0.5 =   -4.76194   Saturation = (a,2 + b,2),0.5 =   8.221343
b = 7.0*(Y-0.847Z)/Y,0.5 =   6.701819   Hue = tanb/a,-1 =   -54.6

Appendix 3.7i. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL 2-naphthoic acid (pH 10.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.388263	50	0.0014	0	0.0065	0.027178	0	0.126185	0
390	0.75568	54.6	0.0042	0.0001	0.0201	0.173288	0.004126	0.829306	0.00546
400	0.89184	82.8	0.0143	0.0004	0.0679	1.055974	0.029538	5.014029	0.03312
410	0.926657	91.5	0.0435	0.0012	0.2074	3.688325	0.101747	17.58526	0.1098
420	0.938386	93.4	0.1344	0.004	0.6456	11.77952	0.350581	56.58378	0.3736
430	0.944904	86.7	0.2839	0.0116	1.3856	23.25798	0.950308	113.5127	1.00572
440	0.94953	104.9	0.3483	0.023	1.7471	34.69265	2.29093	174.0211	2.4127
450	0.953374	117	0.3362	0.038	1.7721	37.50134	4.2387	197.6684	4.446
460	0.956091	117.8	0.2908	0.06	1.6692	32.75209	6.757652	187.9979	7.068
470	0.957671	114.9	0.1954	0.091	1.2876	21.50112	10.01331	141.6829	10.4559
480	0.959962	115.9	0.0956	0.139	0.813	10.63642	15.46506	90.45404	16.1101
490	0.962022	108.8	0.032	0.208	0.4652	3.349374	21.77093	48.69153	22.6304
500	0.963442	109.4	0.0049	0.323	0.272	0.516463	34.04439	28.66896	35.3362
510	0.963747	107.8	0.0093	0.503	0.1582	0.966195	52.25763	16.4357	54.2234
520	0.964424	104.8	0.0633	0.71	0.0782	6.397837	71.76089	7.903805	74.408
530	0.965611	107.7	0.1655	0.862	0.0422	17.21139	89.64481	4.388644	92.8374
540	0.968125	104.4	0.2904	0.954	0.0203	29.35138	96.42292	2.051766	99.5976
550	0.970032	104	0.4334	0.995	0.0087	43.72282	100.3789	0.877685	103.48
560	0.971771	100	0.5945	0.995	0.0039	57.7718	96.69124	0.378991	99.5
570	0.975431	96.3	0.7621	0.952	0.0021	71.58713	89.42521	0.197261	91.6776
580	0.979071	95.8	0.9163	0.87	0.0017	85.94436	81.60165	0.159452	83.346
590	0.97828	88.7	1.0263	0.757	0.0011	89.0556	65.6875	0.095451	67.1459
600	0.979794	90	1.0622	0.631	0.0008	93.66632	55.64248	0.070545	56.79
610	0.980861	89.6	1.0026	0.503	0.0003	88.11368	44.20625	0.026366	45.0688
620	0.981827	87.7	0.8544	0.381	0.0002	73.56915	32.80647	0.017221	33.4137
630	0.982448	83.8	0.6424	0.265	0	52.88824	21.81722	0	22.207
640	0.982483	83.7	0.4479	0.175	0	36.83251	14.39091	0	14.6475
650	0.983553	80	0.2835	0.107	0	22.30699	8.419215	0	8.56
660	0.984763	82.2	0.1649	0.061	0	13.34825	4.937801	0	5.0142
670	0.984521	82.3	0.0874	0.032	0	7.081681	2.592835	0	2.6336
680	0.98601	78.3	0.0468	0.017	0	3.613174	1.312478	0	1.3311
690	0.987084	69.7	0.0227	0.0082	0	1.561755	0.564158	0	0.57154
700	0.988091	71.6	0.0114	0.0041	0	0.806519	0.290064	0	0.29356
710	0.987848	74.3	0.0058	0.0021	0	0.425703	0.154134	0	0.15603
720	0.988889	61.6	0.0029	0.001	0	0.176655	0.060916	0	0.0616
730	0.989202	69.9	0.0014	0.0005	0	0.096803	0.034573	0	0.03495
740	0.989758	75.1	0.0007	0.0002	0	0.052032	0.014866	0	0.01502
750	0.989828	63.6	0.0003	0.0001	0	0.018886	0.006295	0	0.00636
760	0.990976	46.4	0.0002	0.0001	0	0.009196	0.004598	0	0.00464
770	0.991115	66.8	0.0001	0	0	0.006621	0	0	0
780	0.99129	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y = 0.094606

SUM 977.5144 1027.143 1095.439 1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x = 92.47898

Y = K SUM o*s*y = 97.17419

Z = K SUM o*s*z = 103.6354

Y,0.5=   9.857697

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) = 0.315317

Y =Y/(X + Y + Z) = 0.331326

Hunter L, a, b values

L = 10*Y,0.5 =   98.57697

a =17.5*(1.02X-Y)/Y,0.5 =   -5.05173

b = 7.0*(Y-0.847Z)/Y,0.5 =   6.671449

Lightness = L

Saturation = (a,2 + b,2),0.5 =   8.368287

Hue = tanb/a,-1 =   -52.9

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Appendix 4.1. Concentration of sinapine, sinapic acid and thomasidioic acid as a function of time during alkaline hydrolysis and air oxidation of 0.246 mmol/L sinapine solutions in phosphate-boric acid buffers of pH 7, 8.5 and 10 [a].

pH 7				pH 8.5				pH 10			
Time (h)	conc. (mmol/L)			Time (h)	conc. (mmol/L)			Time (h)	conc. (mmol/L)		
	SPN [b]	SA	TA		SPN [b]	SA	TA		SPN [b]	SA	TA
0	0.246	0	0	0	0.246	0	0	0	0.246	0	0
1.92	0.245	nd	nd	1.6	0.242	0.001	nd	1.18	0.22	0.001	nd
23.08	0.231	0.001	nd	23.4	0.159	0.002	0.001	23.78	0.119	0.002	0.001
30.53	0.205	0.001	nd	30.85	0.151	0.002	0.001	31.17	0.093	0.003	0.002
43.88	0.205	0.001	nd	44.25	0.122	0.002	0.001	44.60	0.059	0.009	0.011
49.05	0.198	0.001	nd	49.35	0.109	0.002	0.001	49.70	0.052	0.013	0.013
69.6	0.169	0.001	nd	69.23	0.078	0.001	0.001	69.90	0.035	0.008	0.012
95.49	0.168	0.001	nd	95.19	0.051	0.001	0.004	94.87	0.01	0.001	0.012
335.0	0.087	0.001	0.002								
480.0	0.041	0.002	0.002								

[a] SPN = Sinapine, SA = Sinapic Acid, and TA = Thomasidioic Acid, nd = not detected.
[b] Reaction rate constant k value as in Table 4.1.

Appendix 4.2. Regression output for 1st order and 2nd order reaction fit of concentrations (mmol/L) vs reaction times (hour) of sinapine in phosphate-boric acid buffers of pH 7, 8.5 and 10.

pH 10 1st Order		pH 10 2nd Order	
Constant	-0.0101	Constant	-8665.41
Std Err of Y Est	0.1408	Std Err of Y Est	18639.05
R Squared	0.9846	R Squared	0.7145
No. of Observations	8	No. of Observations	8
Degrees of Freedom	6	Degrees of Freedom	6
X Coefficient(s)	0.0319	X Coefficient(s)	8365.00
Std Err of Coef.	0.0016	Std Err of Coef.	215.73

pH 8.5 1st Order		pH 8.5 2nd Order	
Constant	-0.0013	Constant	2820.88
Std Err of Y Est	0.026	Std Err of Y Est	1402.43
R Squared	0.998	R Squared	0.937
No. of Observations	8	No. of Observations	8
Degrees of Freedom	6	Degrees of Freedom	6
X Coefficient(s)	0.0165	X Coefficient(s)	153.62
Std Err of Coef.	0.0003	Std Err of Coef.	16.26

pH 7 1st Order		pH 7 2nd Order	
Constant	0.0296	Constant	3264.02
Std Err of Y Est	0.0815	Std Err of Y Est	2018.28
R Squared	0.9818	R Squared	0.911
No. of Observations	10	No. of Observations	10
Degrees of Freedom	8	Degrees of Freedom	8
X Coefficient(s)	0.0035	X Coefficient(s)	37.74
Std Err of Coef.	0.0002	Std Err of Coef.	4.16

Appendix 4.3. Effect of time on the Hunter L a b values of 0.246 mmol/L sinapine solutions during alkaline hydrolysis and air oxidation in phosphate-boric acid buffers of pH 7, 8.5 and 10 [a].

Time (h)	Hunter color value		
	L	a	b
	Control [b]		
100	-5.37	5.13	
	pH 7		
0	98.54 ± 0.61^A	-9.37 ± 0.65^A	13.45 ± 0.25^C
24	99.13 ± 0.55^A	-11.37 ± 1.2^A	17.12 ± 1.26^B
108	95.28 ± 1.84^B	-14.5 ± 1.32^B	28.44 ± 1.39^A
	pH 8.5		
0	98.15 ± 0.36^A	-22.43 ± 0.42^B	37.51 ± 1.15^B
24	90.59 ± 1.48^B	-12.89 ± 0.76^A	38.46 ± 0.83^B
108	83.97 ± 1.39^C	-22.54 ± 2.18^B	42.35 ± 1.92^A
	pH 10		
0	97.91 ± 0.75^A	-26.39 ± 1.09^C	46.56 ± 1.69^A
24	79.93 ± 1.38^B	-22.50 ± 1.22^B	36.90 ± 2.53^B
108	73.32 ± 1.81^C	-19.50 ± 1.84^A	33.19 ± 2.45^B

[a] Mean of three replicates±SD. Column values with the same letters were not significantly different ($p \leq 0.05$).
[b] Control = Sinapine in deionized water with a natural pH of 4.3 at zero time.

Appendix 4.4a. ANOVA of Appendix 4.3 (pH 7). The effect of air oxidation time on the color of a 100 μg/mL sinapine in phosphate-boric acid solution of pH 7.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	25.83975556	12.91987778	9.52	0.0138
Error	6	8.14140000	1.35690000		
Corrected Total	8	33.98115556			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	40.16888889	20.08444444	16.67	0.0035
Error	6	7.22786667	1.20464444		
Corrected Total	8	47.39675556			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	366.2604222	183.1302111	154.18	0.0001
Error	6	7.1264667	1.1877444		
Corrected Total	8	373.3868889			

```
Options linesize=78;
Title 'Sinapine in alkaline solution pH 7, Table 4-1-1';
Data;
   Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0        98.61      -9.63      13.45
0        97.89      -9.85      13.2
0        99.1       -8.63      13.7
24       99.7       -11        17.08
24       98.6       -12.71     18.4
24       99.1       -10.4      15.89
108      95.87      -14.63     28.35
108      93.21      -13.12     29.87
108      96.75      -15.76     27.1
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;
```

Appendix 4.4b. ANOVA of Appendix 3.3 (pH 8.5). The effect of air oxidation time on the color of a 100 μg/mL sinapine in phosphate-boric acid solution of pH 8.5.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	301.9180667	150.9590333	106.37	0.0001
Error	6	8.5149333	1.4191556		
Corrected Total	8	310.4330000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	183.9532667	91.9766333	50.05	0.0002
Error	6	11.0251333	1.8375222		
Corrected Total	8	194.9784000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	39.46020000	19.73010000	10.42	0.0112
Error	6	11.35820000	1.89303333		
Corrected Total	8	50.81840000			

```
Options linesize=78;
Title 'Sinapine in alkaline solution pH 8.5, Table 4-1-2';
Data;
  Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0         98.1       -22.4      37.9
0         97.8       -22.0      36.2
0         98.5       -22.9      38.5
24        90.1       -12.8      38.8
24        89.4       -13.7      37.5
24        92.2       -12.2      39.1
108       83.8       -22.3      42.6
108       85.4       -24.8      40.3
108       82.7       -20.5      44.1
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;
```

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Appendix 4.4c. ANOVA of Appendix 4.3 (pH 10).The effect of air oxidation time on the color of a 100 μg/mL sinapine in phosphate-boric acid solution of pH 10.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	971.2246889	485.6123444	254.90	0.0001
Error	6	11.4306000	1.9051000		
Corrected Total	8	982.6552889			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	71.59828889	35.79914444	17.83	0.0030
Error	6	12.04506667	2.00751111		
Corrected Total	8	83.64335556			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	285.6830889	142.8415444	28.04	0.0009
Error	6	30.5621333	5.0936889		
Corrected Total	8	316.2452222			

```
Options linesize=78;
Title 'Sinapine in alkaline solution pH 10, Table 4-1-3';
Data;
   Input Time 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0          97.9      -26.2     46.6
0          97.2      -25.4     44.8
0          98.7      -27.6     48.2
24         80.0      -22.9     36.4
24         81.3      -23.5     34.6
24         78.5      -21.1     39.6
108        73.4      -19.2     34.0
108        71.5      -17.8     30.4
108        75.1      -21.5     35.2
Proc GLM;
   Classes Time;
   Model L a b = Time;
   Means Time / Duncan;
```

Appendix 4.5a. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-100 ug/mL sinapine (pH 7.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.231091	50	0.0014	0	0.0065	0.016176	0	0.075105	0
390	0.333691	54.6	0.0042	0.0001	0.0201	0.076522	0.001822	0.366213	0.00546
400	0.391427	82.8	0.0143	0.0004	0.0679	0.463465	0.012964	2.200648	0.03312
410	0.457815	91.5	0.0435	0.0012	0.2074	1.822217	0.050268	8.687983	0.1098
420	0.552937	93.4	0.1344	0.004	0.6456	6.94099	0.208577	33.34154	0.3736
430	0.668973	86.7	0.2839	0.0116	1.3856	16.48619	0.672799	80.36473	1.00572
440	0.78425	104.9	0.3483	0.023	1.7471	28.6539	1.892161	143.7302	2.4127
450	0.874587	117	0.3362	0.038	1.7721	34.40222	3.888412	181.333	4.446
460	0.930572	117.8	0.2908	0.06	1.6692	31.87789	8.577282	182.98	7.068
470	0.958075	114.9	0.1954	0.091	1.2876	21.51018	10.01754	141.7426	10.4559
480	0.968567	115.9	0.0956	0.139	0.813	10.73176	15.60371	91.26488	16.1101
490	0.972933	108.8	0.032	0.208	0.4652	3.387363	22.01786	49.24379	22.6304
500	0.974678	109.4	0.0049	0.323	0.272	0.522486	34.44141	29.00329	35.3362
510	0.972762	107.8	0.0093	0.503	0.1582	0.975233	52.74646	16.58944	54.2234
520	0.973275	104.8	0.0633	0.71	0.0782	6.456549	72.41943	7.976337	74.408
530	0.972933	107.7	0.1655	0.862	0.0422	17.3419	90.32456	4.421922	92.8374
540	0.973514	104.4	0.2904	0.954	0.0203	29.51477	96.95968	2.063188	99.5976
550	0.973959	104	0.4334	0.995	0.0087	43.89983	100.7853	0.881238	103.48
560	0.972079	100	0.5945	0.995	0.0039	57.79007	96.72182	0.379111	99.5
570	0.970338	96.3	0.7621	0.952	0.0021	71.21336	88.9583	0.196232	91.6776
580	0.962897	95.8	0.9163	0.87	0.0017	86.28019	81.92052	0.160075	83.346
590	0.975637	88.7	1.0263	0.757	0.0011	88.81499	65.51003	0.095193	67.1459
600	0.974267	90	1.0622	0.631	0.0008	93.13797	55.32862	0.070147	56.79
610	0.974164	89.6	1.0026	0.503	0.0003	87.51206	43.90442	0.026186	45.0688
620	0.977627	87.7	0.8544	0.381	0.0002	73.25447	32.66615	0.017148	33.4137
630	0.978761	83.8	0.6424	0.265	0	52.68978	21.73535	0	22.207
640	0.976392	83.7	0.4479	0.175	0	36.60417	14.30169	0	14.6475
650	0.977009	80	0.2835	0.107	0	22.15857	8.363199	0	8.56
660	0.981172	82.2	0.1649	0.061	0	13.29957	4.919791	0	5.0142
670	0.973583	82.3	0.0874	0.032	0	7.002998	2.564027	0	2.6336
680	0.976117	78.3	0.0468	0.017	0	3.576923	1.299309	0	1.3311
690	0.975466	69.7	0.0227	0.0082	0	1.543372	0.557518	0	0.57154
700	0.977937	71.6	0.0114	0.0041	0	0.798231	0.287083	0	0.29356
710	0.976049	74.3	0.0058	0.0021	0	0.420618	0.152293	0	0.15603
720	0.977044	61.6	0.0029	0.001	0	0.174539	0.060186	0	0.0616
730	0.974883	69.9	0.0014	0.0005	0	0.095402	0.034072	0	0.03495
740	0.975534	75.1	0.0007	0.0002	0	0.051284	0.014653	0	0.01502
750	0.974096	63.6	0.0003	0.0001	0	0.018586	0.006195	0	0.00636
760	0.977765	46.4	0.0002	0.0001	0	0.009074	0.004537	0	0.00464
770	0.976151	66.8	0.0001	0	0	0.006521	0	0	0
780	0.974472	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   951.5124 1027.928 977.2103 1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   90.01903
Y = K SUM o*s*y =   97.24842
Z = K SUM o*s*z =   92.45021
Y,0.5=   9.861461

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.321821
Y =Y/(X + Y + Z) =   0.347666

Hunter L, a, b values

L = 10*Y,0.5 =   98.61461
a =17.5*(1.02X-Y)/Y,0.5 =   -9.63424
b = 7.0*(Y-0.847Z)/Y,0.5 =   13.44645

Lightness = L
Saturation = (a,2 + b,2),0.5 =   16.54163
Hue = tanb/a,-1 =   -54.4

Appendix 4.5b. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-
100 ug/mL sinapine (pH 8.5)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.000751	50	0.0014	0	0.0065	5.26E-05	0	0.000244	0
390	0.000749	54.6	0.0042	0.0001	0.0201	0.000172	4.09E-06	0.000822	0.00546
400	0.000836	82.8	0.0143	0.0004	0.0679	0.00099	2.77E-05	0.004701	0.03312
410	0.001761	91.5	0.0435	0.0012	0.2074	0.007007	0.000193	0.03341	0.1098
420	0.006254	93.4	0.1344	0.004	0.6456	0.078511	0.002337	0.377133	0.3736
430	0.030544	86.7	0.2839	0.0116	1.3856	0.751806	0.030718	3.689258	1.00572
440	0.127954	104.9	0.3483	0.023	1.7471	4.675013	0.308715	23.45023	2.4127
450	0.346512	117	0.3362	0.038	1.7721	13.63017	1.54059	71.8442	4.446
460	0.617691	117.8	0.2908	0.06	1.8692	21.15976	4.365838	121.4576	7.068
470	0.818828	114.9	0.1954	0.091	1.2876	18.38388	8.561581	121.1417	10.4559
480	0.919425	115.9	0.0956	0.139	0.813	10.18726	14.81202	86.63436	16.1101
490	0.957839	108.8	0.032	0.208	0.4652	3.334814	21.67629	48.47985	22.6304
500	0.96952	109.4	0.0049	0.323	0.272	0.519721	34.25917	28.84982	35.3362
510	0.969963	107.8	0.0093	0.503	0.1582	0.972427	52.59471	16.54172	54.2234
520	0.971055	104.8	0.0633	0.71	0.0782	6.44182	72.25422	7.958141	74.408
530	0.970577	107.7	0.1655	0.862	0.0422	17.2999	90.10585	4.411214	92.8374
540	0.971737	104.4	0.2904	0.954	0.0203	29.46089	96.78268	2.059422	99.5976
550	0.972284	104	0.4334	0.995	0.0087	43.82432	100.6119	0.879722	103.48
560	0.970748	100	0.5945	0.995	0.0039	57.71094	96.58938	0.378592	99.5
570	0.970134	96.3	0.7621	0.952	0.0021	71.19834	88.93954	0.19619	91.6776
580	0.983346	95.8	0.9163	0.87	0.0017	86.31962	81.95795	0.160148	83.346
590	0.975192	88.7	1.0263	0.757	0.0011	88.77443	65.48012	0.095149	67.1459
600	0.973822	90	1.0622	0.631	0.0008	93.09544	55.30335	0.070115	56.79
610	0.973719	89.6	1.0026	0.503	0.0003	87.47209	43.88436	0.026174	45.0688
620	0.976323	87.7	0.8544	0.381	0.0002	73.15673	32.62256	0.017125	33.4137
630	0.97773	83.8	0.6424	0.265	0	52.63428	21.71246	0	22.207
640	0.975192	83.7	0.4479	0.175	0	36.55918	14.28412	0	14.6475
650	0.975911	80	0.2835	0.107	0	22.13367	8.353802	0	8.56
660	0.980551	82.2	0.1649	0.061	0	13.29116	4.91668	0	5.0142
670	0.972694	82.3	0.0874	0.032	0	6.996605	2.561686	0	2.6336
680	0.974644	78.3	0.0468	0.017	0	3.571523	1.297348	0	1.3311
690	0.974164	69.7	0.0227	0.0082	0	1.541313	0.556774	0	0.57154
700	0.976838	71.6	0.0114	0.0041	0	0.797334	0.28676	0	0.29356
710	0.975157	74.3	0.0058	0.0021	0	0.420234	0.152154	0	0.15603
720	0.975911	61.6	0.0029	0.001	0	0.174337	0.060116	0	0.0616
730	0.973617	69.9	0.0014	0.0005	0	0.095278	0.034028	0	0.03495
740	0.973719	75.1	0.0007	0.0002	0	0.051188	0.014625	0	0.01502
750	0.972523	63.6	0.0003	0.0001	0	0.018556	0.006185	0	0.00636
760	0.976323	46.4	0.0002	0.0001	0	0.00906	0.00453	0	0.00464
770	0.97437	66.8	0.0001	0	0	0.006509	0	0	0
780	0.972557	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   866.7563   1016.925   538.737   1057.013

CIE 1931 tristimulus values

X = K SUM o*s*x =   82.00058
Y = K SUM o*s*y =   96.20751
Z = K SUM o*s*z =   50.96789
Y,0.5=   9.808543

CIE 1931 (X, Y)-chromaticity coordinates

X =X/(X + Y +Z) =   0.357806
Y =Y/(X + Y + Z) =   0.419798

Hunter L, a, b values

L = 10*Y,0.5 =   98.08543
a =17.5*(1.02X-Y)/Y,0.5 =   -22.4214
b = 7.0*(Y-0.847Z)/Y,0.5 =   37.85108

Lightness = L
Saturation = (a,2 + b,2),0.5 =   43.99344
Hue = tanb/a,-1 =   -59.4

Appendix 4.5c. Calculation of the tristimulus X Y Z and L a b values from the % transmittance of the object-
100 ug/mL sinapine (pH 10.0)

nm	o(object)	s(source)	x	y	z	o*s*x	o*s*y	o*s*z	s*y
380	0.000364	50	0.0014	0	0.0065	2.55E-05	0	0.000118	0
390	0.000482	54.6	0.0042	0.0001	0.0201	0.00011	2.63E-06	0.000529	0.00546
400	0.000461	82.8	0.0143	0.0004	0.0679	0.000546	1.53E-05	0.002592	0.03312
410	0.000473	91.5	0.0435	0.0012	0.2074	0.001882	5.19E-05	0.008975	0.1098
420	0.000774	93.4	0.1344	0.004	0.6456	0.009715	0.000289	0.046668	0.3736
430	0.001469	86.7	0.2839	0.0116	1.3856	0.036153	0.001477	0.176446	1.00572
440	0.012783	104.9	0.3483	0.023	1.7471	0.467039	0.030841	2.342707	2.4127
450	0.101194	117	0.3362	0.038	1.7721	3.980514	0.449909	20.98117	4.446
460	0.356815	117.6	0.2908	0.06	1.6692	12.22315	2.521971	70.16122	7.068
470	0.665363	114.9	0.1954	0.091	1.2876	14.93838	6.956971	98.43732	10.4559
480	0.860637	115.9	0.0956	0.139	0.813	9.538112	13.86817	81.11386	16.1101
490	0.942152	108.8	0.032	0.208	0.4652	3.280197	21.32128	47.68587	22.6304
500	0.967649	109.4	0.0049	0.323	0.272	0.518718	34.19303	28.79413	35.3382
510	0.971601	107.8	0.0093	0.503	0.1582	0.974068	52.68348	16.56964	54.2234
520	0.973446	104.8	0.0633	0.71	0.0782	6.457683	72.43215	7.977738	74.408
530	0.97307	107.7	0.1655	0.862	0.0422	17.34433	90.33724	4.422542	92.8374
540	0.974301	104.4	0.2904	0.954	0.0203	29.53863	97.03806	2.064856	99.5976
550	0.97502	104	0.4334	0.995	0.0087	43.94767	100.8951	0.882198	103.48
560	0.973685	100	0.5945	0.995	0.0039	57.88559	96.88168	0.379737	99.5
570	0.973241	96.3	0.7621	0.952	0.0021	71.42634	89.22435	0.196818	91.6776
580	0.988021	95.8	0.9163	0.87	0.0017	86.73002	82.34761	0.160909	83.346
590	0.978658	88.7	1.0263	0.757	0.0011	89.09002	65.71289	0.095488	67.1459
600	0.976941	90	1.0622	0.631	0.0008	93.39356	55.48045	0.07034	56.79
610	0.976357	89.6	1.0026	0.503	0.0003	87.70906	44.00325	0.026244	45.0688
620	0.978796	87.7	0.8544	0.381	0.0002	73.34204	32.70519	0.017168	33.4137
630	0.980793	83.8	0.6424	0.265	0	52.79912	21.78046	0	22.207
640	0.978143	83.7	0.4479	0.175	0	36.66982	14.32735	0	14.6475
650	0.979381	80	0.2835	0.107	0	22.21235	8.383499	0	8.56
660	0.984003	82.2	0.1649	0.061	0	13.33794	4.933985	0	5.0142
670	0.976151	82.3	0.0874	0.032	0	7.021477	2.570792	0	2.6336
680	0.977662	78.3	0.0468	0.017	0	3.582582	1.301365	0	1.3311
690	0.977662	69.7	0.0227	0.0082	0	1.546846	0.558773	0	0.57154
700	0.980035	71.6	0.0114	0.0041	0	0.799944	0.287699	0	0.29356
710	0.977799	74.3	0.0058	0.0021	0	0.421373	0.152566	0	0.15603
720	0.979243	61.6	0.0029	0.001	0	0.174932	0.060321	0	0.0616
730	0.977147	69.9	0.0014	0.0005	0	0.095624	0.034151	0	0.03495
740	0.977456	75.1	0.0007	0.0002	0	0.051385	0.014681	0	0.01502
750	0.976049	63.6	0.0003	0.0001	0	0.018623	0.006208	0	0.00636
760	0.979656	46.4	0.0002	0.0001	0	0.009091	0.004546	0	0.00464
770	0.978315	66.8	0.0001	0	0	0.006535	0	0	0
780	0.976872	63.4	0	0	0	0	0	0	0

K = 100/SUM s * y =   0.094606   SUM   841.5812 1013.502 382.6153 1057.013

CIE 1931 tristimulus values
X = K SUM o*s*x =   79.61885
Y = K SUM o*s*y =   95.88362
Z = K SUM o*s*z =   36.1978
Y,0.5=   9.792018

CIE 1931 (X, Y)-chromaticity coordinates
X =X/(X + Y +Z) =   0.376092
Y =Y/(X + Y + Z) =   0.452922

Hunter L, a, b values
L = 10*Y,0.5 =   97.92018
a =17.5*(1.02X-Y)/Y,0.5 =   -26.2221
b = 7.0*(Y-0.847Z)/Y,0.5 =   46.62661

Lightness = L
Saturation = (a,2 + b,2),0.5 =   53.49427
Hue = tanb/a,-1 =   -60.6
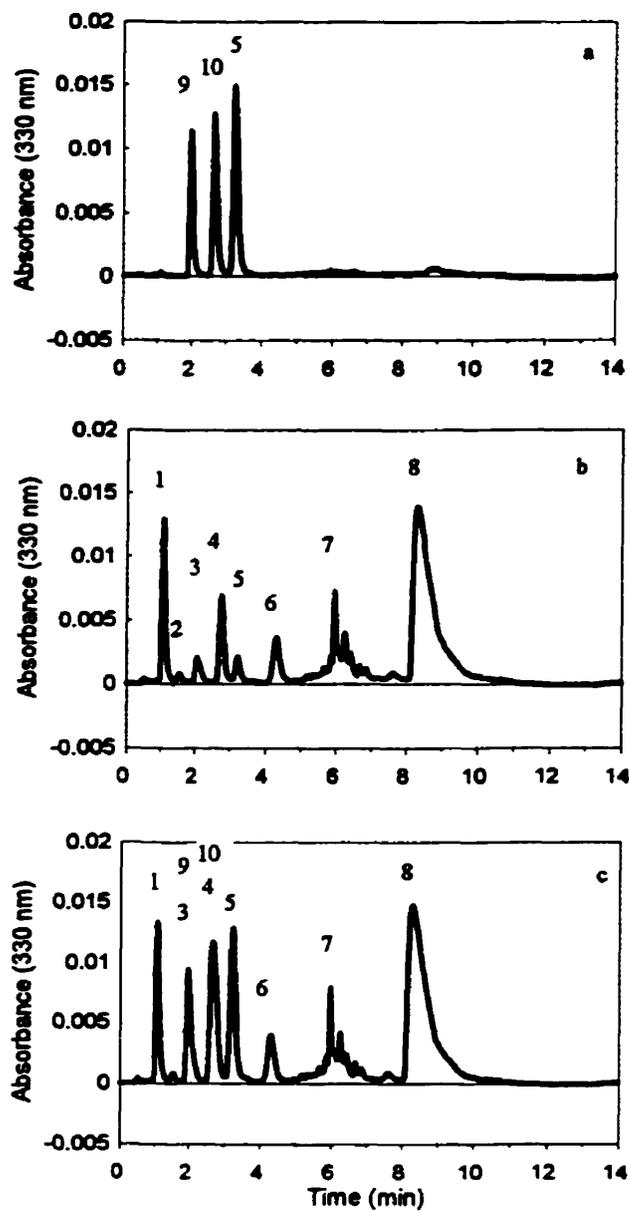
Appendix 5.1a-b. Chromatograms (by gradient A) of (a) standard phenolic acids, (b) methanol extract of canola flour and (c) the mixture of standards and the methanol extract. Peak identifications: 5. sinapic acid, 8. sinapine, 9. ferulic acid, 10. p-coumaric, other peaks were unidentified.

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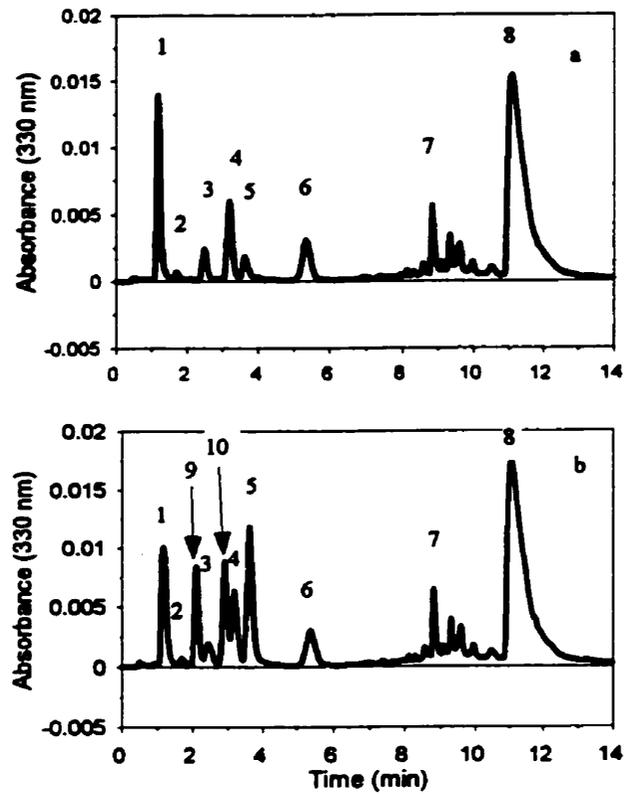
Appendix 5.2a-b. Chromatograms (by gradient B) of (a) methanol extract of canola flour and (b) the mixture of standards and the methanol extract. Peak identifications: 5. sinapic acid, 8. sinapine, 9. ferulic acid, 10. p-coumaric, other peaks were unidentified.

Appendix 5.3. ANOVA of Table 5.1. Effect of extraction conditions on the phenolic content of canola flour. SPNB = sinapine bisulfate, SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPNB

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	8.26213949	2.75404650	3.90	0.0489
Error	9	6.35625926	0.70625103		
Corrected Total	12	14.61839877			

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	4.79542930	1.59847643	3.90	0.0489
Error	9	3.68923520	0.40991502		
Corrected Total	12	8.48466450			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.04205943	0.01401981	6.66	0.0144
Error	8	0.01684137	0.00210517		
Corrected Total	11	0.05890080			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	105.9145309	35.3048436	9.76	0.0047
Error	8	28.9394427	3.6174303		
Corrected Total	11	134.8539736			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	131.3671463	43.7890488	5.89	0.0201
Error	8	59.4933796	7.4366724		
Corrected Total	11	190.8605259			

Options linesize=78;
Title 'Effect of extraction conditions on phenolic content, Table 5-1';
Data;
   Input Condition 1-9 SPNB 11-19 SPN 21-29 SA 31-39 TH 41-49 TF 51-59;
Cards;

```
1          14.624585 11.141686 0.2908617 27.532352 29.246308
1          13.967468 10.641064 0.3588511 26.795615 25.883412
1          14.144094 10.775625 0.4094255 31.096128 31.396998
                              0.4068085 26.846687
2          13.887383 10.580051 0.2775    27.466253 25.766102
2          14.099596 10.741725 0.3768191 24.447813 27.799481
2          13.966702 10.64048  0.3666277 26.41831  29.95017
3          14.564245 11.095716 0.4668404 33.385683 31.792126
3          15.751904 12.00053  0.4811489 31.02143  35.908937
3          16.587936 12.637458 0.5245532 32.071076 37.85707
3          16.54883  12.607665
4          16.328766 12.44001  0.4256489 34.471076 31.357894
4          15.989734 12.18172  0.421117  32.388806 36.089411
4          13.917766 10.603199 0.3689574 36.107955 36.441342
Proc GLM;
  Classes Condition;
  Model SPNB SPN SA TH TF = Condition;
  Means Condition / Duncan;

Note:  1 = 50 °C, 10 min; 2 = 75 °C, 20 min: 3 = refluxing 20 min, 1-3, 100% methanol;
       4 = 75 °C, 20 min, 70% methanol.
```

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Appendix 5.3. Peak areas in relation to concentrations of standard sinapine and sinapic acid (2 μL of sinapine and 1 μL sinapic acid solutions) [a].

Concentration (μg/mL)	Sinapine	Sinapic Acid
200	4981317 ± 216039^A	4179471 ± 341659^A
100	2664600 ± 238650^B	2043461 ± 157815^B
50	1227633 ± 56052^C	1029987 ± 83265^C
25	575705 ± 51077^D	518170 ± 43115^D

[a] Mean of six determinations±SD. Column values with the same letters were not significantly different ($p \leq 0.05$).

Appendix 5.5a. ANOVA of Table 5.2 and Appendix 5.4. Time and area as a function of sinapine concentration.

General Linear Models Procedure

Dependent Variable: TIME

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.16940850	0.38980283	45.73	0.0001
Error	20	0.17046200	0.00852310		
Corrected Total	23	1.33987050			

Dependent Variable: AREA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	6.85801E+13	2.28600E+13	836.01	0.0001
Error	20	5.46887E+11	2.73444E+10		
Corrected Total	23	6.91270E+13			

```
Options linesize=78;
Title 'Time and area as a function of conc of sinapine, Table 5-1-1';
Data;
   Input conc 1-9 Time 11-19 Area 21-29;
Cards;
200      7.914      5058512
200      7.934      5091104
200      7.965      5284429
200      7.799      4702799
200      7.747      4983973
200      7.705      4767082
100      8.05       3044549
100      8.108      2817664
100      8.135      2698511
100      7.885      2416946
100      7.919      2546974
100      7.905      2462955
50       8.312      1200661
50       8.293      1222911
50       8.292      1322047
50       8.215      1215870
50       8.204      1154152
50       8.21       1250157
25       8.445      507545
25       8.423      636630
25       8.432      520305
25       8.306      592428
25       8.55       586750
25       8.33       610573
Proc GLM;
  Classes conc;
  Model Time Area = conc;
  Means conc / Duncan;
```

Appendix 5.5b. ANOVA of Table 5.2 and Appendix 5.4. Time and area as a function of sinapic acid concentration.

General Linear Models Procedure

Dependent Variable: TIME

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.10233960	0.03411320	1.57	0.2349
Error	16	0.34699560	0.02168723		
Corrected Total	19	0.44933520			

Dependent Variable: AREA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3.93781E+13	1.31260E+13	349.03	0.0001
Error	16	6.01713E+11	3.76071E+10		
Corrected Total	19	3.99798E+13			

```
Options linesize=78;
Title 'Time and area as a function of conc. of sinapic acid, Table 5-1-2';
Data;
  Input conc 1-9 Time 11-19 Area 21-29;
Cards;
200      3.669      3963918
200      3.711      3807272
200      3.791      4615016
200      3.773      4054769
200      3.590      4456378
100      3.339      2020181
100      3.399      1843689
100      3.740      2121856
100      3.745      2261671
100      3.436      1969906
50       3.591      932517
50       3.355      959437
50       3.719      1050727
50       3.784      1072175
50       3.578      1135080
25       3.443      455464
25       3.403      543869
25       3.696      491229
25       3.506      544111
25       3.616      556177
Proc GLM;
  Classes conc;
  Model Time Area = conc;
  Means conc / Duncan;
```

Appendix 6.1. ANOVA of Table 6.1. The effect of procedure on the color and protein contents of canola protein isolate.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2257.942500	752.647500	974.30	0.0001
Error	8	6.180000	0.772500		
Corrected Total	11	2264.122500			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	22.50000000	7.50000000	41.67	0.0001
Error	8	1.44000000	0.18000000		
Corrected Total	11	23.94000000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	161.6025000	53.8675000	138.12	0.0001
Error	8	3.1200000	0.3900000		
Corrected Total	11	164.7225000			

Dependent Variable: PC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	495.8318250	165.2772750	754.43	0.0001
Error	8	1.7526000	0.2190750		
Corrected Total	11	497.5844250			

```
Options linesize=78;
Title 'Procedure effect on the color and protein content, Table 6-1';
Data;
  Input Proced 1-9 L 11-19 a 21-29 b 31-39 PC 41-49;
Cards;
1      71.9      -1.7      13.9      77.20
1      71.3      -2.8      14.3      78.96
1      71.9      -3.0      15.6      78.35
2      60.4      -1.6      9.6       78.00
2      60.1      -1.1      10.2      78.31
2      58.6      -1.2      10.5      78.02
3      49.8      0.5       20.6      66.06
3      49.2      0.7       19.6      66.18
3      47.4      1.2       19.5      65.91
4      34.9      0.8       11.6      64.62
4      33.7      0.5       12.3      64.42
4      34.9      0.5       12.4      64.76
Proc GLM;
  Classes Proced;
  Model L a b PC = Proced;
  Means Proced / Duncan;
```

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Appendix 6.2. ANOVA of Table 6.2. The effect of procedures on the phenolic contents of canola protein isolate. SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3.67942500	1.22647500	23.80	0.0002
Error	8	0.41220000	0.05152500		
Corrected Total	11	4.09162500			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00356667	0.00118889	1.00	0.4420
Error	8	0.00953333	0.00119167		
Corrected Total	11	0.01310000			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	144.1400250	48.0466750	144.64	0.0001
Error	8	2.6574000	0.3321750		
Corrected Total	11	146.7974250			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	297.7230000	99.2410000	113.51	0.0001
Error	8	6.9944000	0.8743000		
Corrected Total	11	304.7174000			

```
Options linesize=78;
Title 'Procedure effect on phenolic contents, Table 6-2';
Data;
  Input Proced 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49;
Cards;
1     0.4     0.14    3.84    5.85
1     0.36    0.23    2.67    5.42
1     0.59    0.18    2.34    5.74
2     1.07    0.18    2.35    6.03
2     0.52    0.17    2.21    6.56
2     0.63    0.24    2.1     7.93
3     1.68    0.24    8.43    13.17
3     1.82    0.21    8.3     15.1
3     1.36    0.18    7.93    13.82
4     1.73    0.23    10.1    18.19
4     1.97    0.25    11.32   18.54
4     1.52    0.21    9.84    16.25
Proc GLM;
```

```
Classes Proced;
Model SPN SA TH TF = Proced;
Means Proced / Duncan;
```

Note: 1 = AAP, 2 = PMM, 3 = BAP (pH 8.5) and 4 = BAP (pH 10), abbreviations see Table 6.2.

Appendix 6.3a. ANOVA of Table 6.3. The effect of time on the color of canola flour (whole seed) during autoclaving.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	606.6600000	202.2200000	3370.33	0.0001
Error	8	0.4800000	0.0600000		
Corrected Total	11	607.1400000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	65.24250000	21.74750000	2174.75	0.0001
Error	8	0.08000000	0.01000000		
Corrected Total	11	65.32250000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.18000000	0.06000000	2.40	0.1433
Error	8	0.20000000	0.02500000		
Corrected Total	11	0.38000000			


```
Options linesize=78;
Title 'Time effect on the color of whole seed flour during autoclaving, Table 6-3-1';
Data;
  Input Time 4-6 L 8-11 a 13-15 b 17-20;
Cards;
   0    70.3 2.2 17.3
   0    70.2 2.1 17.4
   0    70.1 2.0 17.2
   15   59.1 5.0 17.2
   15   58.8 5.2 17.4
   15   58.8 5.1 17.3
   30   54.4 7.1 17.1
   30   55.1 7.0 17.3
   30   54.9 6.9 17.2
   45   51.4 8.4 16.9
   45   51.5 8.2 17.3
   45   51.0 8.3 16.8
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;
```

Appendix 6.3b. ANOVA of Table 6.3. The effect of time on the color of canola flour (dehulled) during autoclaving.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1060.342500	353.447500	2019.70	0.0001
Error	8	1.400000	0.175000		
Corrected Total	11	1061.742500			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	100.1025000	33.3675000	2224.50	0.0001
Error	8	0.1200000	0.0150000		
Corrected Total	11	100.2225000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.75000000	0.25000000	1.56	0.2725
Error	8	1.28000000	0.16000000		
Corrected Total	11	2.03000000			

```
Options linesize=78;
Title 'Time effect on the color of dehulled flour during autoclaving, Table 6-3-2';
Data;
  Input Time 4-6 L 8-11 a 13-15 b 17-20;
Cards;
   0   79.2 0.6 20.4
   0   79.3 0.5 19.0
   0   79.1 0.4 19.7
  15   63.1 5.0 19.3
  15   62.9 5.0 19.4
  15   63.0 4.7 19.2
  30   58.1 6.8 19.5
  30   58.6 6.6 19.4
  30   58.5 6.7 19.3
  45   53.6 8.3 18.9
  45   54.9 8.2 19.4
  45   55.0 8.1 18.7
Proc GLM;
  Classes Time;
  Model L a b = Time;
  Means Time / Duncan;
```

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Appendix 6.4a. ANOVA of Table 6.4. The effect of time on the phenolic contents of canola flour (whole seed) during autoclaving. SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent, SAIN = sinapic acid released from insoluble-bound phenolics, THIN = total insoluble phenolics determined by HPLC, TFIN = total insoluble phenolic determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	9.67582500	3.22527500	11.25	0.0031
Error	8	2.29440000	0.28680000		
Corrected Total	11	11.97022500			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.01380000	0.00460000	1.10	0.4031
Error	8	0.03340000	0.00417500		
Corrected Total	11	0.04720000			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	359.8298250	119.9432750	55.40	0.0001
Error	8	17.3196000	2.1649500		
Corrected Total	11	377.1494250			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	242.0522250	80.6840750	34.53	0.0001
Error	8	18.6948000	2.3368500		
Corrected Total	11	260.7470250			

Dependent Variable: SAIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00622500	0.00207500	4.15	0.0477
Error	8	0.00400000	0.00050000		
Corrected Total	11	0.01022500			

Dependent Variable: THIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.06870000	0.02290000	1.92	0.2042
Error	8	0.09520000	0.01190000		
Corrected Total	11	0.16390000			

Dependent Variable: TFIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	3	13.46910000	4.48970000	7.66	0.0097
Error	8	4.66600000	0.58600000		
Corrected Total	11	18.15710000			

```
Options linesize=78;
Title 'Time effect on phenolic contents during autoclaving, whole seed flour, Table 6-4-
1';
Data;
  Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49 SAIN 51-59 THIN 61-69 TFIN 71-79;
Cards;
0        11.88    0.44    33.1    34.57    0.09    1.73    4.85
0        12.32    0.41    33.2    35.22    0.06    1.76    4.02
0        12.07    0.62    31.2    34.01    0.09    1.64    5.38
15       11.04    0.45    21.05   27.82    0.11    2.01    6.56
15       10.34    0.43    20.1    24.73    0.14    1.69    5.32
15       11.53    0.47    22.3    25.12    0.11    1.76    6.84
30       10.32    0.39    23.04   27.61    0.16    1.98    6.84
30       11.43    0.39    19.5    24.15    0.12    1.81    7.78
30       10.02    0.48    18.72   23.84    0.11    1.76    6.23
45       9.08     0.41    19.21   23.05    0.17    1.93    8.5
45       9.79     0.37    17.49   23.82    0.13    1.96    7.2
45       9.87     0.42    18.98   21.23    0.12    1.87    7.1
Proc GLM;
  Classes Time;
  Model SPN SA TH TF SAIN THIN TFIN = Time;
  Means Time / Duncan;
```

Appendix 6.4b. ANOVA of Table 6.4. The effect of time on the phenolic contents of canola
flour (dehulled) during autoclaving. SPN = sinapine, SA = sinapic acid, TH = total
phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent,
SAIN = sinapic acid released from insoluble-bound phenolics, THIN = total insoluble
phenolics determined by HPLC, TFIN = total insoluble phenolic determined by Folin-
Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	35.63482500	11.87827500	15.04	0.0012
Error	8	6.31700000	0.78962500		
Corrected Total	11	41.95182500			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.37080000	0.12360000	25.48	0.0002
Error	8	0.03880000	0.00485000		
Corrected Total	11	0.40960000			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

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Model	3	478.0152000	159.3384000	29.86	0.0001
Error	8	42.6902000	5.3362750		
Corrected Total	11	520.7054000			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	160.4238000	53.4746000	15.40	0.0011
Error	8	27.7710000	3.4713750		
Corrected Total	11	188.1948000			

Dependent Variable: SAIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.79462500	0.26487500	75.14	0.0001
Error	8	0.02820000	0.00352500		
Corrected Total	11	0.82282500			

Dependent Variable: THIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.52602500	0.50867500	48.91	0.0001
Error	8	0.08320000	0.01040000		
Corrected Total	11	1.60922500			

Dependent Variable: TFIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	40.03042500	13.34347500	15.43	0.0011
Error	8	6.91740000	0.86467500		
Corrected Total	11	46.94782500			

```
Options linesize=78;
Title 'Time effect on phenolic contents during autoclaving, deholled flour, Table 6-4-2';
Data;
   Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49 SAIN 51-59 THIN 61-69 TFIN 71-79;
Cards;
0        15.99    0.67    42.56    42.28    0.06    1.04    2.88
0        15.32    0.85    41.23    43.45    0.1     1.13    3.86
0        16.21    0.67    44.73    46.51    0.06    0.98    2.98
15       12.69    0.45    29.13    39.15    0.63    1.79    7.19
15       13.69    0.39    32.31    37.21    0.72    1.74    7.85
15       11.87    0.45    29.1     39.89    0.57    1.87    5.12
30       11.36    0.27    26.13    37.75    0.72    1.91    7.04
30       13.45    0.38    30.54    38.54    0.67    1.94    8.23
30       12.12    0.28    31.23    35.67    0.56    1.76    6.75
45       12.22    0.36    29.42    36.43    0.75    2.1     8.28
45       11.12    0.26    25.46    33.24    0.73    1.82    8.45
45       10.23    0.25    24.32    32.12    0.68    1.87    7.12
Proc GLM;
  Classes Time;
  Model SPN SA TH TF SAIN THIN TFIN = Time;
  Means Time / Duncan;
```

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Appendix 6.5a. ANOVA of Table 6.5 (1hour). The effect of phenolic during basic extraction (1h) on the color of canola protein isolate.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	21.38000000	10.69000000	188.65	0.0001
Error	6	0.34000000	0.05666667		
Corrected Total	8	21.72000000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.62000000	0.31000000	18.60	0.0027
Error	6	0.10000000	0.01666667		
Corrected Total	8	0.72000000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.74000000	0.37000000	2.92	0.1301
Error	6	0.76000000	0.12666667		
Corrected Total	8	1.50000000			

```
Options linesize=78;
Title 'Phenolic effect (1h) on the color, Table 6-5-1';
Data;
   Input Phenol 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0        63.9        -1.9        12.5
0        64.3        -1.6        12.7
0        63.8        -1.6        12.9
1        67.9        -2.3        13.6
1        67.6        -2.1        13.0
1        67.6        -2.2        12.7
2        66.2        -1.7        12.8
2        66.7        -1.5        12.3
2        66.6        -1.6        12.1
Proc GLM;
  Classes Phenol;
  Model L a b = Phenol;
  Means Phenol / Duncan;
```

Note: 0 = No phenolic, 1 = 1% Sinapine and 2 = 1% Sinapic acid.

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Appendix 6.5b. ANOVA of Table 6.5 (5hour). The effect of phenolic during basic extraction (5h) on the color of canola protein isolate.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	2.00000000	1.00000000	30.00	0.0008
Error	6	0.20000000	0.03333333		
Corrected Total	8	2.20000000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.06000000	0.03000000	1.50	0.2963
Error	6	0.12000000	0.02000000		
Corrected Total	8	0.18000000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.18000000	0.09000000	1.42	0.3125
Error	6	0.38000000	0.06333333		
Corrected Total	8	0.56000000			

```
Options linesize=78;
Title 'Phenolic effect (5h) on the color, Table 6-5-2';
Data;
  Input Phenol 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0        64.7        -1.7        11.9
0        64.7        -1.6        11.6
0        64.4        -1.5        11.6
1        65.8        -2          11.8
1        65.5        -1.8        11.8
1        65.5        -1.6        11.5
2        64.6        -1.7        11.8
2        64.8        -1.8        11.3
2        64.4        -1.6        11.1
Proc GLM;
  Classes Phenol;
  Model L a b = Phenol;
  Means Phenol / Duncan;
```

Note: 0 = No phenolic, 1 = 1% sinapine and 2 = 1% sinapic acid.

Appendix 6.5c. ANOVA of Table 6.5 (24hour). The effect of phenolics during basic extraction (24h) on the color of canola protein isolate.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	16.74000000	8.37000000	86.59	0.0001
Error	6	0.58000000	0.09666667		
Corrected Total	8	17.32000000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.02000000	0.01000000	0.60	0.5787
Error	6	0.10000000	0.01666667		
Corrected Total	8	0.12000000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.14000000	0.07000000	2.62	0.1517
Error	6	0.16000000	0.02666667		
Corrected Total	8	0.30000000			

```
Options linesize=78;
Title 'Phenolic effect (24h) on the color, Table 6-5-3';
Data rcb;
   Input Phenol 1-9 L 11-19 a 21-29 b 31-39;
Cards;
0        63.4        -1.6        10.7
0        63.4        -1.5        10.5
0        63.1        -1.7        10.3
1        64.5        -1.6        10.4
1        64.0        -1.7        10.2
1        65.0        -1.5        10.3
2        66.5        -1.6        10.4
2        66.7        -1.6        10.1
2        66.6        -1.3        10.1
Proc GLM;
  Classes Phenol;
  Model L a b = Phenol;
  Means Phenol / Duncan;
```

Note: 0 = No phenolic, 1 = 1% sinapine and 2 = 1% sinapic acid.

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Appendix 6.5d. ANOVA of Table 6.5. The effect of the interaction between time and sinapine (phenol) on the color of canola protein isolate.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	35.80500000	7.16100000	97.65	0.0001
Error	12	0.88000000	0.07333333		
Corrected Total	17	36.68500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PHENOL	1	17.40500000	17.40500000	237.34	0.0001
TIME	2	11.61000000	5.80500000	79.16	0.0001
PHENOL*TIME	2	6.79000000	3.39500000	46.30	0.0001

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.82500000	0.16500000	9.00	0.0009
Error	12	0.22000000	0.01833333		
Corrected Total	17	1.04500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PHENOL	1	0.24500000	0.24500000	13.36	0.0033
TIME	2	0.39000000	0.19500000	10.64	0.0022
PHENOL*TIME	2	0.19000000	0.09500000	5.18	0.0239

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	19.06000000	3.81200000	63.53	0.0001
Error	12	0.72000000	0.06000000		
Corrected Total	17	19.78000000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PHENOL	1	0.02000000	0.02000000	0.33	0.5744
TIME	2	18.76000000	9.38000000	156.33	0.0001
PHENOL*TIME	2	0.28000000	0.14000000	2.33	0.1393

Options linesize=78;
Title 'Interaction effect of time and 1% sinapine, Table 6-5-4';
Data;
    Input Phenol 1-9 L 11-19 a 21-29 b 31-39 Time 41-49;
Cards;

0	63.9	-1.9	12.5	1
0	64.3	-1.6	12.7	1
0	63.8	-1.6	12.9	1
1	67.9	-2.3	13.6	1
1	67.6	-2.1	13.0	1

```
1        67.6      -2.2      12.7      1
0        64.7      -1.7      11.9      5
0        64.7      -1.6      11.6      5
0        64.4      -1.5      11.6      5
1        65.8      -2        11.8      5
1        65.5      -1.8      11.8      5
1        65.5      -1.6      11.5      5
0        63.4      -1.6      10.7      24
0        63.4      -1.5      10.5      24
0        63.1      -1.7      10.3      24
1        64.5      -1.6      10.4      24
1        64        -1.7      10.2      24
1        65        -1.5      10.3      24
Proc GLM;
  Classes Phenol Time;
  Model L a b = Phenol Time Phenol*Time;
  Lsmeans Phenol Time Phenol*Time /stderr;
  Means Phenol Time / Duncan;
```

Appendix 6.5e. ANOVA of Table 6.5. The effect of the interaction between time and sinapic acid (phenol) on the color of canola protein isolate.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	26.98000000	5.39600000	129.50	0.0001
Error	12	0.50000000	0.04166667		
Corrected Total	17	27.48000000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PHENOL	1	16.82000000	16.82000000	403.68	0.0001
TIME	2	1.27000000	0.63500000	15.24	0.0005
PHENOL*TIME	2	8.89000000	4.44500000	106.68	0.0001

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.08500000	0.01700000	1.02	0.4481
Error	12	0.20000000	0.01666667		
Corrected Total	17	0.28500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PHENOL	1	0.00500000	0.00500000	0.30	0.5939
TIME	2	0.04000000	0.02000000	1.20	0.3349
PHENOL*TIME	2	0.04000000	0.02000000	1.20	0.3349

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	14.96500000	2.99300000	44.89	0.0001
Error	12	0.80000000	0.06666667		
Corrected Total	17	15.76500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PHENOL	1	0.40500000	0.40500000	6.07	0.0298
TIME	2	14.56000000	7.28000000	109.20	0.0001
PHENOL*TIME	2	0.00000000	0.00000000	0.00	1.0000

```
Options linesize=78;
Title 'Interaction effect of time and sinapic acid, Table 6-5-5';
Data;
  Input Phenol 1-9 L 11-19 a 21-29 b 31-39 Time 41-49;
Cards;
0        63.9      -1.9      12.5       1
0        64.3      -1.6      12.7       1
0        63.8      -1.6      12.9       1
1        66.2      -1.7      12.8       1
1        66.7      -1.5      12.3       1
1        66.6      -1.6      12.1       1
0        64.7      -1.7      11.9       5
0        64.7      -1.6      11.6       5
0        64.4      -1.5      11.6       5
1        64.6      -1.7      11.8       5
1        64.8      -1.8      11.3       5
1        64.4      -1.6      11.1       5
0        63.4      -1.6      10.7      24
0        63.4      -1.5      10.5      24
0        63.1      -1.7      10.3      24
1        66.5      -1.6      10.4      24
1        66.7      -1.6      10.1      24
1        66.6      -1.3      10.1      24
Proc GLM;
  Classes Phenol Time;
  Model L a b = Phenol Time Phenol*Time;
  Lsmeans Phenol Time Phenol*Time /stderr;
  Means Phenol Time / Duncan;
```

Note: For phenol, 0 = no phenolic and 1 = 1% sinapic acid.

Appendix 6.6a. ANOVA of Table 6.6 (no phenolic). The effect of time on the retained phenolic contents during basic extraction for control (no phenolic). SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.02010000	0.00670000	19.14	0.0005
Error	8	0.00280000	0.00035000		
Corrected Total	11	0.02290000			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00180000	0.00060000	29.27	0.0001
Error	8	0.00016400	0.00002050		
Corrected Total	11	0.00196400			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.76940000	0.58980000	55.38	0.0001
Error	8	0.08520000	0.01065000		
Corrected Total	11	1.85460000			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	63.87480000	21.29160000	555.19	0.0001
Error	8	0.30680000	0.03835000		
Corrected Total	11	64.18160000			

```
Options linesize=78;
Title 'Basic extraction phenolic content, no phenolic control, Table 6-6-1';
Data;
  Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49;
Cards;
0        0.07     0        1.16     6.12
0        0.03     0        1.02     6.12
0        0.02     0        0.76     5.46
1        0        0        0        0
1        0        0        0        0
1        0        0        0        0
5        0        0.013    0.13     0.91
5        0        0.008    0.08     0.79
5        0        0.009    0.12     0.82
24       0.13     0.035    0.22     1.2
24       0.09     0.035    0.21     1.15
24       0.08     0.02     0.26     1.07
Proc GLM;
  Classes Time;
  Model SPN SA TH TF = Time;
  Means Time / Duncan;
```

Appendix 6.6b. ANOVA of Table 6.6 (1% sinapine). The effect of time on the retained phenolic contents during basic extraction for isolate with 1% sinapine bisulfate. SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	86.92582500	28.97527500	781.00	0.0001

```
Error                        8      0.29680000      0.03710000
Corrected Total             11     87.22262500
```

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.76042500	0.25347500	506.95	0.0001
Error	8	0.00400000	0.00050000		
Corrected Total	11	0.76442500			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	127.2858000	42.4286000	372.70	0.0001
Error	8	0.9107380	0.1138422		
Corrected Total	11	128.1965380			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	233.9402250	77.9800750	133.44	0.0001
Error	8	4.6752000	0.5844000		
Corrected Total	11	238.6154250			

```
Options linesize=78;
Title 'Basic extraction phenolic content, 1%SPN, Table 6-6-2';
Data;
   Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49;
Cards;
0        8.51      0        11.8      14.33
0        8.85      0        11.21     16.23
0        8.74      0        11.01     16.54
1        3.45      0        4.14      5.43
1        3.75      0        3.45      5.76
1        3.24      0        3.96      5.34
5        2.91      0.41     4.32      6.22
5        2.72      0.42     3.78      5.81
5        2.56      0.46     3.57      5.49
24       1.84      0.52     3.807     4.25
24       1.81      0.57     3.733     5.89
24       1.57      0.59     3.62      5.46
Proc GLM;
  Classes Time;
  Model SPN SA TH TF = Time;
  Means Time / Duncan;
```

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Appendix 6.6c. ANOVA of Table 6.6 (1% sinapic acid). The effect of time on the retained phenolic contents during basic extraction for isolate with 1% sinapic acid. SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00202500	0.00067500	27.00	0.0002
Error	8	0.00020000	0.00002500		
Corrected Total	11	0.00222500			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	126.4047000	42.1349000	200.28	0.0001
Error	8	1.6830000	0.2103750		
Corrected Total	11	128.0877000			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	103.8950250	34.6316750	110.94	0.0001
Error	8	2.4974000	0.3121750		
Corrected Total	11	106.3924250			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	148.9694250	49.6564750	122.89	0.0001
Error	8	3.2326000	0.4040750		
Corrected Total	11	152.2020250			

Options linesize=78;
Title 'Basic extraction phenolic content, 1%SA, Table 6-6-3';
Data;
  Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49;
Cards;

0	0	10.38	12.22	14.16
0	0	11.2	10.57	13.26
0	0	9.98	12.31	15.48
1	0	5.25	4.86	5.93
1	0	4.12	4.98	6.21
1	0	4.52	4.35	5.62
5	0	4.16	5.33	7.47
5	0	3.64	4.85	6.84
5	0	3.54	4.97	6.45
24	0.04	1.91	5.17	5.73
24	0.02	1.85	5.12	5.98
24	0.03	1.67	4.56	5.78

Proc GLM;
  Classes Time;
  Model SPN SA TH TF = Time;
  Means Time / Duncan;

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Appendix 6.7a. ANOVA of Table 6.7 (15 min). The effect of phenolics on the color of canola protein isolate during autoclaving for 15 min.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	2.78000000	1.39000000	24.53	0.0013
Error	6	0.34000000	0.05666667		
Corrected Total	8	3.12000000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.38000000	0.19000000	19.00	0.0025
Error	6	0.06000000	0.01000000		
Corrected Total	8	0.44000000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1.82000000	0.91000000	19.50	0.0024
Error	6	0.28000000	0.04666667		
Corrected Total	8	2.10000000			

```
Options linesize=78;
Title 'The effect of phenolic on color of protein isolate, Table 6-7-1';
Data;
  Input Phenol 1-5 L 7-10 a 12-14 b 17-20;
Cards;
0      52.4 0.6   15.1
0      52.5 0.4   15.3
0      52.0 0.5   14.9
1      51.4 0.8   14.9
1      51.5 0.7   15.5
1      51.0 0.6   15.2
2      52.7 0.3   16.0
2      52.7 0.2   16.1
2      52.4 0.1   16.2
Proc GLM;
  Classes Phenol;
  Model L a b = Phenol;
  Means Phenol / Duncan;
```

Note: 0 = No phenolic, 1 = 1% Sinapine and 2 = 1% Sinapic acid.

Appendix 6.7b. ANOVA of Table 6.7 (30 min). The effect of phenolics on the color of canola protein isolate during autoclaving for 30 min.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	10.58000000	5.29000000	27.84	0.0009
Error	6	1.14000000	0.19000000		
Corrected Total	8	11.72000000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.32000000	0.16000000	9.60	0.0135
Error	6	0.10000000	0.01666667		
Corrected Total	8	0.42000000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	5.18000000	2.59000000	28.78	0.0008
Error	6	0.54000000	0.09000000		
Corrected Total	8	5.72000000			

```
Options linesize=78;
Title 'The effect of phenolic on color of protein isolate, Table 6-7-2';
Data;
  Input Phenol 1-5 L 10-13 a 15-18 b 20-23;
Cards;
0       50.4 -0.3 14.2
0       50.9 -0.1 13.7
0       49.6 -0.2 14.7
1       47.7 0.3  14
1       48.2 0.3  14.1
1       48.1 0    13.9
2       50.4 -0.2 15.7
2       50.5 -0.3 15.8
2       50.0 -0.1 15.6
Proc GLM;
  Classes Phenol;
  Model L a b = Phenol;
  Means Phenol / Duncan;
```

Note: 0 = NO phenolic, 1 = 1% Sinapine and 2 = 1% Sinapic acid.

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Appendix 6.7c. ANOVA of Table 6.7 (45 min). The effect of phenolics on the color of canola protein isolate during autoclaving for 45 min.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	10.94000000	5.47000000	82.05	0.0001
Error	6	0.40000000	0.06666667		
Corrected Total	8	11.34000000			

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.38000000	0.19000000	6.33	0.0332
Error	6	0.18000000	0.03000000		
Corrected Total	8	0.56000000			

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	3.78000000	1.89000000	51.55	0.0002
Error	6	0.22000000	0.03666667		
Corrected Total	8	4.00000000			

```
Options linesize=78;
Title 'The effect of phenolic on color of protein isolate, Table 6-7-3';
Data;
   Input Phenol 1-5 L 10-13 a 15-18 b 20-23;
Cards;
0       48.5 0.8   14.7
0       48.5 0.7   14.8
0       48.2 0.6   14.6
1       47.1 1.1   14.2
1       47.5 0.7   14.7
1       46.7 1.2   14.3
2       49.9 0.5   16.1
2       49.8 0.6   15.8
2       49.7 0.4   15.8
Proc GLM;
  Classes Phenol;
  Model L a b = Phenol;
  Means Phenol / Duncan;
```

Note: 0 = NO phenolic, 1 = 1% Sinapine and 2 = 1% Sinapic acid.

Appendix 6.7d. ANOVA of Table 6.7. The effect of the interaction between sinapine (SPN) and time on the color of canola protein isolate during autoclaving.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	62.74000000	12.54800000	90.71	0.0001
Error	12	1.66000000	0.13833333		
Corrected Total	17	64.40000000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
SPN	1	10.58000000	10.58000000	76.48	0.0001
TIME	2	50.77000000	25.38500000	183.51	0.0001
SPN*TIME	2	1.39000000	0.69500000	5.02	0.0260

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.72500000	0.54500000	23.36	0.0001
Error	12	0.28000000	0.02333333		
Corrected Total	17	3.00500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
SPN	1	0.40500000	0.40500000	17.36	0.0013
TIME	2	2.29000000	1.14500000	49.07	0.0001
SPN*TIME	2	0.03000000	0.01500000	0.64	0.5430

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3.54000000	0.70800000	9.04	0.0009
Error	12	0.94000000	0.07833333		
Corrected Total	17	4.48000000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
SPN	1	0.08000000	0.08000000	1.02	0.3322
TIME	2	3.33000000	1.66500000	21.26	0.0001
SPN*TIME	2	0.13000000	0.06500000	0.83	0.4597

Options linesize=78;
Title 'Interaction effect between time and sinapine on the color, Table 6-7-4';
Data;
  Input SPN 1-5 Time 7-8 L 10-13 a 15-18 b 20-23;
Cards;
0   15 52.4 0.6   15.1
0   15 52.5 0.4   15.3
0   15 52   0.5   14.9
1   15 51.4 0.8   14.9
1   15 51.5 0.7   15.5
1   15 51.0 0.6   15.2
0   30 50.4 -0.3 14.2

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```
0     30 50.9 -0.1 13.7
0     30 49.6 -0.2 14.7
1     30 47.7 0.3  14
1     30 48.2 0.3  14.1
1     30 48.1 0    13.9
0     45 48.5 0.8  14.7
0     45 48.5 0.7  14.6
0     45 48.2 0.6  14.6
1     45 47.1 1.1  14.2
1     45 47.5 0.7  14.7
1     45 46.7 1.2  14.3
Proc GLM;
   Classes SPN Time;
   Model L a b = SPN Time SPN*Time;
   Lsmeans SPN Time SPN*Time / stderr;
   Means SPN Time / Duncan;
```

Appendix 6.7e. ANOVA of Table 6.7. The effect of the interaction between sinapic acid (SA) and time on the color of canola protein isolate during autoclaving.

General Linear Models Procedure

Dependent Variable: L

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	37.64500000	7.52900000	70.58	0.0001
Error	12	1.28000000	0.10666667		
Corrected Total	17	38.92500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
SA	1	1.44500000	1.44500000	13.55	0.0031
TIME	2	34.57000000	17.28500000	162.05	0.0001
SA*TIME	2	1.63000000	0.81500000	7.64	0.0072

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.20500000	0.44100000	44.10	0.0001
Error	12	0.12000000	0.01000000		
Corrected Total	17	2.32500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
SA	1	0.12500000	0.12500000	12.50	0.0041
TIME	2	2.01000000	1.00500000	100.50	0.0001
SA*TIME	2	0.07000000	0.03500000	3.50	0.0635

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	8.30500000	1.66100000	28.47	0.0001
Error	12	0.70000000	0.05833333		
Corrected Total	17	9.00500000			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
SA	1	6.84500000	6.84500000	117.34	0.0001

TIME		2	1.27000000	0.63500000	10.89	0.0020
SA*TIME		2	0.19000000	0.09500000	1.63	0.2367

```
Options linesize=78;
Title 'Interaction effect between time and sinapic acid on the color, Table 6-7-5';
Data;
  Input SA 1-5 Time 7-8 L 10-13 a 15-18 b 20-23;
Cards;
0     15 52.4 0.6   15.1
0     15 52.5 0.4   15.3
0     15 52.0 0.5   14.9
1     15 52.7 0.3   16.0
1     15 52.7 0.2   16.1
1     15 52.4 0.1   16.2
0     30 50.4 -0.3  14.2
0     30 50.9 -0.1  13.7
0     30 49.6 -0.2  14.7
1     30 50.4 -0.2  15.7
1     30 50.5 -0.3  15.8
1     30 50.0 -0.1  15.6
0     45 48.5 0.8   14.7
0     45 48.5 0.7   14.8
0     45 48.2 0.6   14.6
1     45 49.9 0.5   16.1
1     45 49.8 0.6   15.8
1     45 49.7 0.4   15.8
Proc GLM;
  Classes SA Time;
  Model L a b = SA Time SA*Time;
  Lsmeans SA Time SA*Time / stderr;
  Means SA Time / Duncan;
```

Appendix 6.8a. ANOVA of Table 6.8 (no phenolic). The effect of time on the phenolic contents of canola protein isolates during autoclaving for control (no phenolic). SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent, SAIN = sinapic acid released from insoluble-bound phenolics, THIN = total insoluble phenolics determined by HPLC, TFIN = total insoluble phenolics determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source		DF	Sum of Squares	Mean Square	F Value	Pr > F
Model		3	0.15930000	0.05310000	88.17	0.0001
Error		8	0.00481800	0.00060225		
Corrected Total		11	0.16411800			

Dependent Variable: SA

Source		DF	Sum of Squares	Mean Square	F Value	Pr > F
Model		3	0.00180000	0.00060000	13.65	0.0016
Error		8	0.00035168	0.00004396		
Corrected Total		11	0.00215168			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.98430000	0.32810000	26.73	0.0002
Error	8	0.09820000	0.01227500		
Corrected Total	11	1.08250000			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3.08422500	1.02807500	12.48	0.0022
Error	8	0.65920000	0.08240000		
Corrected Total	11	3.74342500			

Dependent Variable: SAIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00060000	0.00020000	10.30	0.0040
Error	8	0.00015528	0.00001941		
Corrected Total	11	0.00075528			

Dependent Variable: THIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.06022500	0.02007500	20.59	0.0004
Error	8	0.00780000	0.00097500		
Corrected Total	11	0.06802500			

Dependent Variable: TFIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3.95482500	1.31827500	10.46	0.0038
Error	8	1.00860000	0.12607500		
Corrected Total	11	4.96342500			

```
Options linesize=78;
Title 'Phenolic contents after autoclaving , no phenolic, Table 6-8-1';
Data;
  Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49 SAIN 51-59 THIN 61-69 TFIN 71-79;
Cards;
0      0.07     0        1.16    6.12    0        0.1     3.29
0      0.03     0        1.02    6.12    0        0.11    2.98
0      0.02     0        0.76    5.46    0        0.09    3.03
15     0.35     0.045    0.7     4.99    0.007    0.22    3.86
15     0.31     0.025    0.82    4.97    0.011    0.23    4.98
15     0.36     0.02     0.67    5.34    0.012    0.18    4.12
30     0.093    0        0.44    4.7     0.0082   0.26    4.29
30     0.12     0        0.49    5.3     0.0098   0.26    4.65
30     0.087    0        0.42    4.7     0.012    0.23    4.32
45     0.13     0.0092   0.21    4.52    0.029    0.35    4.37
45     0.09     0.011    0.21    4.65    0.018    0.25    4.3
45     0.08     0.0098   0.24    4.36    0.013    0.27    4.86
Proc GLM;
```

```
Classes Time;
Model SPN SA TH TF SAIN THIN TFIN = Time;
Means Time / Duncan;
```

Appendix 6.8b. ANOVA of Table 6.8 (1% sinapine bisulfate). The effect of time on the
phenolic contents of canola protein isolates during autoclaving for isolate with 1%
sinapine bisulfate. SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by
HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent, SAIN = sinapic acid
released from insoluble-bound phenolics, THIN = total insoluble phenolics determined by
HPLC, TFIN = total insoluble phenolics determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	20.48700000	6.82900000	25.04	0.0002
Error	8	2.18160000	0.27270000		
Corrected Total	11	22.66860000			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00322500	0.00107500	20.38	0.0004
Error	8	0.00042200	0.00005275		
Corrected Total	11	0.00364700			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	60.23242500	20.07747500	116.75	0.0001
Error	8	1.37580000	0.17197500		
Corrected Total	11	61.60822500			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	79.98000000	26.66000000	45.80	0.0001
Error	8	4.65680000	0.58210000		
Corrected Total	11	84.63680000			

Dependent Variable: SAIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.61582500	0.20527500	241.50	0.0001
Error	8	0.00680000	0.00085000		
Corrected Total	11	0.62262500			

Dependent Variable: THIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2.00940000	0.66980000	61.73	0.0001
Error	8	0.08680000	0.01085000		

```
Corrected Total          11        2.09620000
```

Dependent Variable: TFIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	16.43460000	5.47820000	42.80	0.0001
Error	8	1.02400000	0.12800000		
Corrected Total	11	17.45860000			

```
Options linesize=78;
Title 'Phenolic contents after autoclaving, 1% Sinapine bisulfate, Table 6-8-2';
Data;
  Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49 SAIN 51-59 THIN 61-69 TFIN 71-79;
Cards;
0        8.51     0        11.8     14.33    0.02     0.2      3.99
0        8.85     0        11.21    16.23    0        0.13     4.02
0        8.74     0        11.01    16.54    0.01     0.12     4.35
15       6.23     0        7.2      10.31    0.43     0.79     6.61
15       6.12     0        6.92     11.32    0.42     0.76     6.94
15       5.89     0        6.43     9.87     0.35     0.61     6.13
30       6.15     0.004    6.6      10.41    0.53     0.94     6.52
30       6.12     0.01     6.12     9.65     0.54     0.79     7.34
30       5.34     0.016    6.78     9.34     0.49     0.76     6.75
45       4.28     0.055    5.95     9.05     0.59     1.35     6.58
45       5.98     0.035    4.97     9.43     0.58     1.42     7.14
45       5.67     0.03     5.64     9.12     0.63     1.13     7.21
Proc GLM;
  Classes Time;
  Model SPN SA TH TF SAIN THIN TFIN = Time;
  Means Time / Duncan;
```

Appendix 6.8c. ANOVA of Table 6.8 (1% sinapic acid). The effect of time on the phenolic contents of canola protein isolates during autoclaving for isolate with 1% sinapic acid. SPN = sinapine, SA = sinapic acid, TH = total phenolic determined by HPLC, TF = total phenolic determined by Folin-Ciocalteu reagent, SAIN = sinapic acid released from insoluble-bound phenolics, THIN = total insoluble phenolics determined by HPLC, TFIN = total insoluble phenolics determined by Folin-Ciocalteu reagent.

General Linear Models Procedure

Dependent Variable: SPN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.05130000	0.01710000	22.06	0.0003
Error	8	0.00620000	0.00077500		
Corrected Total	11	0.05750000			

Dependent Variable: SA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	153.6927000	51.2309000	392.20	0.0001
Error	8	1.0450000	0.1306250		
Corrected Total	11	154.7377000			

Dependent Variable: TH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	147.8996250	49.2998750	140.98	0.0001
Error	8	2.7976000	0.3497000		
Corrected Total	11	150.6972250			

Dependent Variable: TF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	131.8971000	43.9657000	41.00	0.0001
Error	8	8.5788000	1.0723500		
Corrected Total	11	140.4759000			

Dependent Variable: SAIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.94710000	0.31570000	237.82	0.0001
Error	8	0.01062000	0.00132750		
Corrected Total	11	0.95772000			

Dependent Variable: THIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	4.32908025	1.44302675	259.92	0.0001
Error	8	0.04441400	0.00555175		
Corrected Total	11	4.37349425			

Dependent Variable: TFIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	15.70642500	5.23547500	40.37	0.0001
Error	8	1.03760000	0.12970000		
Corrected Total	11	16.74402500			

```
Options linesize=78;
Title 'Phenolic contents after autoclaving, 1% Sinapic acid, Table 6-8-3';
Data;
   Input Time 1-9 SPN 11-19 SA 21-29 TH 31-39 TF 41-49 SAIN 51-59 THIN 61-69 TFIN 71-79;
Cards;
0        0        10.38    12.22    14.16    0.005    0.016    4.32
0        0        11.2     10.57    13.26    0.013    0.011    4.33
0        0        9.98     12.31    15.48    0.012    0.012    3.98
15       0        6.57     6.94     9.2      0.35     0.83     6.5
15       0        6.12     6.21     9.87     0.35     0.74     6.72
15       0        6.78     6.68     11.23    0.32     0.68     7.45
30       0.07     3.67     4.67     8.65     0.64     1.07     6.83
30       0.07     3.65     4.98     9.23     0.63     0.86     7.56
30       0.04     3.42     5.71     8.67     0.53     0.92     6.97
45       0.22     0.84     2.13     3.44     0.79     1.74     5.88
45       0.12     0.84     1.92     5.92     0.74     1.74     6.02
45       0.14     0.93     1.89     5.67     0.72     1.62     6.43
Proc GLM;
  Classes Time;
  Model SPN SA TH TF SAIN THIN TFIN = Time;
  Means Time / Duncan;
```

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